Editorial introductions

Current Opinion in Rheumatology was launched in 1989. It is one of a successful series of review journals whose unique format is designed to provide a systematic and critical assessment of the literature as presented in the many primary journals. The field of Rheumatology is divided into 15 sections that are reviewed once a year. Each section is assigned a Section Editor, a leading authority in the area, who identifies the most important topics at that time. Here we are pleased to introduce the Journal's Editor in Chief and Section Editors for this issue.

EDITOR IN CHIEF

Steven B. Abramson

Steven B. Abramson, MD, is Senior Vice President and Vice Dean for education, faculty and academic affairs at NYU Langone Medical Center, USA. He is the Frederick H. King Professor and Chair of the Department of Medicine. As Vice Dean, he oversees the implementation of the medical school's nationally recognized curriculum for the 21st Century, including the country's first multispeciality three-year pathway to the MD degree.

A graduate of Dartmouth College, Dr Abramson earned his MD from Harvard Medical School and trained at NYU Medical Center and Bellevue Hospital, USA. He served as the Director of the Division of Rheumatology from 2000–2013, and has had numerous leadership positions in academic medicine. He has served on the Board of the National Arthritis Foundation, as Co-Editor of *Arthritis & Rheumatism*, a member of the Rheumatology Board of the American Board of Internal Medicine (ABIM), President of the Osteoarthritis Research Society International (OARSI), and former chairman of the Arthritis Advisory Committee of the Food and Drug Administration (FDA).

Dr Abramson has extensive experience in both basic science and clinical research in the field of inflammation and arthritis, and has published more than 300 papers on these and related topics. He received the prestigious American College of Rheumatology Distinguished Basic Investigator Award in 2011.

SECTION EDITORS

Hasan Yazici

Hasan Yazici is a retired Professor of Medicine and Rheumatology. He currently practices rheumatology, part time, at the Academic Hospital in Istanbul, Turkey. He still attends the weekly dedicated Behçet's syndrome outpatient clinic he started with a group of his colleagues 40 years ago, and co-edits a journal on controversy in Rheumatology, *Letter to Editor/Clinical* and *Experimental Rheumatology*.

After he received his MD from the University of Istanbul in 1969, he trained in internal medicine and rheumatology at University of Nebraska and Creighton University (Metabolic Research Unit) in Omaha, Nebraska, USA, where his mentor was Paul D. Saville. After returning to Turkey in 1974 he joined Cerrahpasa Medical Faculty of the University of Istanbul where he started both the multidisciplinary Behçet Disease Outpatient Clinic and the Division of Rheumatology, which he chaired until his retirement 5 years ago. His main research interests are Behçet's syndrome, clinical research methodology, and ethics. He has published many original articles in peer reviewed journals in addition to his text book contributions, editorials, and reviews. Being the most cited author on Behçet's disease on Web of Science, he has recieved a number of prestigious awards and has a long list of memberships in scientific societies which includes being a member of the Science Academy (Turkey), European Academy of Sciences, Master of the American College of Rheumatology and the recipient of the 2012 EULAR award for Meritorious Service in Rheumatology.

Yusuf Yazici

Yusuf Yazici, MD, is a Clinical Associate Professor of Medicine at the New York University School of Medicine, USA. Dr Yazici is also the Director of the Seligman Center for Advanced Therapeutics at the NYU Hospital for Joint Diseases, and Director of the Behcet's Syndrome Evaluation, Treatment and Research Center at NYU Hospital for Joint Diseases.

Dr Yazici earned his medical degree from Cerrahpasa Medical Faculty, University of Istanbul, Turkey. He completed his internship and residency at Creighton University in Nebraska, USA, and his fellowship in rheumatology at the Hospital for Special Surgery of Weill Medical College of Cornell University, USA.

His areas of interest are rheumatoid arthritis, early arthritis, patient reported outcomes, database and registry management and monitoring of arthritis patients in regard to clinical response and adverse events related to treatment and Behcet's syndrome. He has published over 200 articles and presented at various national and international meetings over 100 times.

He divides his time between seeing patients and running the Seligman Center, conducting both industry and investigator initiated trials in the areas of RA and Behcet's syndrome.

Jose U. Scher

Dr Jose U. Scher is Assistant Professor of Medicine at the New York University School of Medicine; Director of the NYU-HJD Arthritis Clinic and Director of the NYU Psoriatic Arthritis Center, USA.

Dr Scher was born and raised in Buenos Aires, Argentina, where he received his medical degree and training in clinical immunology at the National Genetic Databank under the mentorship of Ana Di Lonardo. He moved to New York in the early 2000's and graduated from an internal medicine residency and rheumatology fellowship at New York University School of Medicine. After joining the laboratory of Dan Littman as a fellow, he became faculty in the Division of Rheumatology at NYU-Langone Hospital for Joint Diseases.

Over the last several years his research has focused on the role of the human microbiome (the totality of microorganisms and their genes residing in the human body) as determinant of autoimmunity and rheumatic diseases. Mentored by Drs Steven Abramson, Dan Littman, Gerald Weissmann and Marty Blaser, Dr Scher helped establish (and now direct) the Microbiome Center for Rheumatology and Autoimmunity (MiCRA). His investigations have led to the observation of oral and gut microbiome community alterations (dysbiosis) in patients with rheumatoid and psoriatic arthritis. His current research focus is understanding how changes in mucosal microbiome modulate distal inflammatory responses and/or serve as markers for immunomodulation.

Dr Scher receives funding from the NIH and the Arthritis Foundation, has written extensively on the field of microbiome, and has lectured nationally and internationally on the pathogenesis of rheumatoid and psoriatic arthritis.

Mukundan Attur

Mukundan Attur, PhD, is Associate Professor of Medicine in the Department of Medicine, Division of Rheumatology, of NYU School of Medicine, NYU Langone Health, New York, NY, USA. He received his PhD degree from Madurai Kamaraj University, India, and joined the NYU Division of Rheumatology. Dr Attur's interests are in the expression and role of inflammatory mediators in osteoarthritic cartilage, with the aim to elucidate the autocrine and paracrine mechanisms of action of inflammatory cytokines and lipids (eicosanoids) in chondrocytes using genomics and proteomics approaches. Dr Attur is privileged to work under the supervision of Dr Steven B. Abramson, with whom he has developed transcriptome and protein-based biomarkers to identify subjects at risk for development of severe knee osteoarthritis. Currently, as Director of the Rheumatology Research Laboratory at NYU Hospital for Joint Diseases, Dr Attur has established and maintained the arthritis biobank. His current research focuses on the pathophysiology, diagnosis, and treatment of osteoarthritis, with special interest in extracellular non-collagenous proteins expressed in bone and cartilage. Dr Attur, through a proteogenomics-system biology approach, has identified several genes and proteins that are now examined as biomarkers to predict knee osteoarthritis development and progression. He is the author of over 69 publications and numerous invited reviews.

Vasculitis 2018: the bench and the bedside

Hasan Yazici[a] *and Yusuf Yazici*[b]

The systemic vasculitides remain difficult to classify, recognize and manage. The main obstacle is our inadequate knowledge of disease mechanisms and this year's review opens with two excellent basic science chapters that address this very issue.

The Epigenetics in Vasculitis (Coit *et al.* pp. 4–15) begins with a useful review for the clinician of the three main epigenetic mechanisms, namely DNA methylation, histone changes and none coding RNAs, in which case the best studied mechanism concerns miRNAs. It continues with what we know about epigenetic aberrations in antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). Very broadly, there is good evidence for aberrations in all three of the mentioned epigenetic mechanisms in AAV. We now know that there is a considerable delay in silencing of the genes *PRTN3* and *MPO*, responsible for the production of PR3 and MPO in AAV, antibodies to which are the main biomarkers for AAV. This delay can, in turn, be because of more than one epigenetic aberration, like histone and DNA methylation changes or over-expression of other genes, leading to over or under expression of *PRTN3* and *MPO* [1,2].

In another systemic vasculitis, giant cell arteritis (GCA), there is also good evidence from temporal artery biopsies for hypomethylated gene loci involved in inflammation-regulating pathways [3]. Of these, the authors point out, the calcineurin–NFAT pathway can be clinically important as dipyridamole, a clinically commonly used calcineurin inhibitor, apparently also inhibits the production of important inflammatory cytokines, like IFN-gamma, IL-6 and IL-17, in a lupus mice model [4]. Coit *et al.* [5] underline that miRNAs are probably also important in GCA arteritis as well, by highlighting a recent study reporting abundance of miRNAs in the tissue specimens of elderly patients with GCA. In this study, no association of these miRNAs with any gene expression has been proposed, and there apparently is evidence that the presence of these miRNAs has to do with senescence and tissue repair. We propose that the Croci *et al.* [5] study is particularly praiseworthy as the authors also study tissue specimens from patients with diseases other than GCA. The inclusion of diseased controls in epigenetic studies seems to be regrettably infrequent, as has been formally noted, also for genetic association studies [6].

In the next chapter, Söderberg and Segelmark (pp. 16–23) nicely summarize our current understanding on how NETosis is important in vascular injury. Apparently, this involves a number of direct, like inducing apoptosis in endothelial cells, and indirect, like causing immune complex and autoantibody formation, mechanisms. They also bring forward recent data about how NETosis important in drug-induced vasculitis [7] and AAV [8]. Their catchy title, on the other hand, underlines that this important tool of innate immunity apparently initially described in body's fight against microbial agents can also be protective, for example, in helping to avoid mucosal injury in Behçet's disease [9]. Having said all this, the authors also rightfully warn the reader that currently, there is no clinically useful method to detect NETosis. They underline this as related to the discussion of using NETs as biomarkers whereas we propose that the issues of reproducibility and sensitivity are also important in understanding disease mechanisms and potential use of new molecular findings in ultimately formulating new drugs.

The ensuing chapter on usefulness of PET imaging in the diagnosis of large vessel vasculitis (LVV; Pipitone *et al.*, pp. 24–29) informs us that the addition of PET to CT or MRI imaging improves the clinical usefulness of these imaging modalities. They advise us to remember that these techniques are useful mainly in GCA or Takayasu arteritis because of the larger vessel sizes involved. Although they are particularly useful in detecting clinically not apparent but pathologically involved vessel segments, they are not much helpful in follow-up because of the following reasons: the isotope uptake by the vessel wall begins to rapidly decrease days after

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Curr Opin Rheumatol 2018, 30:1–3

DOI:10.1097/BOR.0000000000000462

treatment begins and an increased isotope uptake in the vessel wall during the disease course may not be because of an exacerbation but to the healing process.

The authors refer us to a recent article that shows that semiquantitative image interpretations are superior to visual assessment only [10] and the overall conclusion is that although PET is a useful modality in recognizing LVV, especially GCA, more needs to be done in improving scoring techniques.

In daily practice, we usually understand a hematologic–chemical–immunologic or genetic–epigenetic measurement whenever we talk about a biomarker. Hatemi *et al.* begin their chapter on Biomarkers in Vasculitis (pp. 30–35) by reminding us of the National Institutes of Health (NIH) definition of a biomarker [11], which includes imaging studies. This justifies us to conclude that the initial three chapters of this annual review are specific topics in biomarkers, whereas the fourth chapter is an overall view. Among a list of important issues, which we learn or reminded of are that after many years of continuing debate that anti-PR3 levels are most probably important both in assessing disease progression [12] or response to treatment [13]; we now have a promise of a urine test to assess renal disease in AAV [14]. The authors conclude their chapter with the remark that 'a lot of work' needs to be done for better, more useful biomarkers in vasculitis. We like to add that better disease definitions in vasculitis are also needed for better biomarkers as we are reminded by an article which highlighted that autoantibody type was more important than clinical diagnosis in predicting relapses in AAV [15].

The next chapter (Argyropoulou *et al.*, pp. 36–43) is about atheromatosis and arteriosclerosis in peak systolic velocity (PSV). The authors rightfully point out that there are no hard data to help us with the atherogenic profile of these patients at the onset, for that matter unfortunately during the course, of their disease and we usually ascribe the ensuing accelerated atherosclerosis to the long-term use of glucocorticoids, frequent presence of kidney disease and, obviously, to the inflammation of the vascular wall itself. They quote a comprehensive recent review [16] on the state-of-the-art at-hand about two biomarkers for atheromatosis (internal medial thickness and plaque presence, both assessed by ultrasound) and one biomarker for arteriosclerosis (carotid–femoral pulse wave velocity). Apparently, these tools are quite useful for assessing cardiovascular risk but not necessarily useful in judging treatment affects. The authors continue with recent, mainly confirmatory data about increased cardiovascular risk in GCA, Takayasu arteritis, AAV, Kawasaki and Behçet's disease. They specifically underline that, even though former reports proposed no appreciably increased cardiovascular risk in Behçet's disease, more recent work suggests otherwise [17]. We differ from the authors and propose that there still are no hard data to show appreciably increased cardiovascular mortality in Behçet's disease and the often-quoted presence of subclinical atherosclerosis rather likely does not translate into frank cardiovascular disease in this condition. The most compelling clinical evidence for this is the data from Cerrahpasa group, where they had shown that the mortality in Behçet's disease actually decreased with the passage of time [18] and no evidence of increased frequency of clinical ischemic heart disease is observed in a controlled study among patients with Behçet's disease [19]).

An increase in malignancies in AAV has been appreciated for some time, and the review by Trejo *et al.* (pp. 44–49) gives us a clear perspective of the current considerations. It is gratifying to note that the incidence of malignancies in AAV is decreasing as the use of cyclophosphamide is more and more replaced by rituximab [20]. In fact, recent data [21] indicate that the risk of malignancies in AAV is comparable with that in the general population, perhaps with the exception of nonmelanoma skin cancers.

The final chapter in this annual review is about the current management of vasculitis in Behçet's disease (Merashli *et al.*, pp. 50–56), and it is clear that biologics are being used more and more with apparently better patient outcomes. As the authors bring up the current consensus is still more leaning towards not anticoagulating in thrombosis of Behçet's disease. The newly and importantly described changes in fibrin structure in Behçet's disease surely gives more weight to this practice [22]. On the other hand, continuing lack of controlled clinical trials that address this issue is simply regrettable.

In brief, we have covered some bench and some bedside in this annual review of vasculitis. Although the topics we have attempted to cover are obviously limited, we strongly suspect that there are indeed ways to go between the two, and we can begin to more confidently talk about from the bench to the bedside in the wide field of vasculitides.

Acknowledgements

None.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

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An update on the role of epigenetics in systemic vasculitis

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Purpose of review
The purpose of this review is to discuss recent observations of epigenetic changes related to the complex pathogenesis of systemic vasculitides and their contribution to the field.

Recent findings
There have been new observations of epigenetic changes in vasculitis and their potential role in disease pathogenesis in antineutrophil cytoplasmic antibody-associated vasculitis, giant-cell arteritis, Kawasaki disease, Behçet's disease, and IgA vasculitis. Some of this recent work has focused on the efficacy of using DNA methylation and miRNA expression as clinical biomarkers for disease activity and how DNA methylation and histone modifications interact to regulate disease-related gene expression.

Summary
DNA methylation, histone modification, and miRNA expression changes are all fruitful ground for biomarker discovery and therapeutic targets in vasculitis. Current knowledge has provided targeted and suggested effects, but in many cases, has relied upon small cohorts, cosmopolitan cell populations, and limited knowledge of functional interactions. Expanding our knowledge of how these epigenetic mechanisms interact in a disease-specific and cell-specific manner will help to better understand the pathogenesis of systemic vasculitis.

Keywords
DNA methylation, epigenetics, histone modification, microRNA, systemic vasculitis

INTRODUCTION

Systemic vasculitides are a heterogeneous group of complex inflammatory diseases of unknown cause. They are characterized by histological evidence of leukocyte infiltration, inflammation, and necrosis of the vessel wall and vascular occlusion [1]. Numerous genetic loci have been associated with increased risk of vasculitis, with human leukocyte antigen (HLA) genes encoding major histocompatibility complex (MHC) proteins being most robust and pointing toward the importance of the immune system in pathogenesis [2]. Environmental risk factors include exposure to silica dust, unknown viral or bacterial infections, drugs, farming occupations as well as complex factors like age [3–6]. The contribution of genetics alone to the pathogenesis of a systemic vasculitis varies with manifestations, but does not account for the entirety of the risk. Epigenetic mechanisms governing gene expression sit at the interface of genetic and environmental factors related to a variety of diseases [7]. Epigenetics is the study of hereditary, phenotypic traits that can alter the chromosome without changing the underlying genetic sequence [8]. DNA methylation, histone modifications, and noncoding RNA are epigenetic mechanisms that control gene expression and regulate cellular development, differentiation and activity (extensively reviewed in [9▪]).

DNA methylation is an epigenetic mechanism that consists of the addition of a methyl group to cytosines, primarily within CpG dinucleotides, catalyzed by DNA methyltransferases (DNMTs). De-novo DNA methylation is conducted primarily by DNMT3A and DNMT3B which are essential during the gestational development of mammals. Although DNMT1 is primarily responsible for maintaining established methylation patterns from cell to cell,

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Curr Opin Rheumatol 2018, 30:4–15
DOI:10.1097/BOR.0000000000000451

KEY POINTS

- Epigenetic characterization in vasculitis can provide insights into disease pathogenesis and identify novel potential therapeutic targets.
- The autoantigen gene loci in ANCA-associated vasculitis *PRTN3* and *MPO* undergo epigenetic changes that correlate with disease activity and with PR3 and MPO expression.
- Epigenetic changes and microRNA expression profiles can be developed into disease biomarkers in vasculitis, but larger cohorts and validation studies are needed.
- Cell-type specific epigenetic signatures can be used to map causal variants in genetic risk loci and identify genetic-epigenetic interactions in vasculitis.

the extent to which their functions overlap is still being explored [10]. DNMT function and targeting are regulated by complex systems including DNMT expression levels, RNA molecules, amino-acid residue modifications, genomic sequence and methylation status, histone tail signatures, transcription factor availability, and chromatin accessibility [10]. DNMT1 is also capable of recruiting histone deacetylase 1 (HDAC1) which promotes the formation of heterochromatin through the removal of acetyl groups from histone proteins, silencing gene expression and providing a link between DNA methylation and histone modification [11].

Histone modification is an epigenetic mechanism that regulates the dynamic chromatin structure and subsequently gene expression [12]. The functional opposition to HDACs, histone acetyltransferases (HATs), promote the formation of euchromatin that is permissive to protein-DNA interactions [12]. Acetylation is only one of a multitude of histone modifications found in the genome which can include ubiquitylation, residue-specific methylation, and phosphorylation [13]. Identifying patterns of histone modifications and their relationship to gene expression has provided a way to understand how chromatin structure controls the regulation of cellular functions, and has led to the identification of 'bivalent chromatin' that contains both permissive (e.g., H3K4me2) and repressive (e.g., H3K27me3) histone modifications poised for expression depending on cellular requirements, which is vital during cell development [9▪].

Noncoding RNAs are transcribed but not translated into proteins, and act as an epigenetic mechanism to regulate gene expression. The most studied are microRNAs (miRNAs), around 22 nucleotides in length that regulate posttranscriptional gene silencing through translational control of mRNA molecules. miRNAs target the 3′ untranslated region of their target mRNA molecule and control their stability and protein interactions [14]. miRNA expression is controlled by other epigenetic mechanisms and itself controls these mechanisms as an 'epigenetics-miRNA regulatory circuit' that, when perturbed, can contribute to disease pathogenesis [15].

The focus of this review is to discuss our current knowledge of the role epigenetics in systemic vasculitis, and more specifically to highlight new developments in the field of interest to clinicians and researchers.

ANTINEUTROPHIL CYTOPLASMIC ANTIBODY-ASSOCIATED VASCULITIS

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a systemic necrotizing vasculitis of small vessels characterized by the presence of autoantigens against neutrophil cytoplasmic proteins, specifically myeloperoxidase (MPO) and proteinase 3 (PR3) [1,16]. The presence of antineutrophil cytoplasmic antibodies against MPO and PR3 is used in the classification of AAV, though not all patients have ANCA. ANCAs have been implicated in vascular damage in AAV patients. Neutrophils in AAV patients are more sensitive to activation by ANCA, as demonstrated by the production of reactive oxygen species and neutrophil extracellular traps (NETs) [17,18]. In normal neutrophils, MPO and PR3 expression primarily occurs early in cell development, contributing to the formation of intracellular granules, but AAV cells continue to express MPO and PR3 into maturity which indicates a deviation from normal gene silencing [19,20].

Ciavatta *et al.*, Yang *et al.*, and Jones *et al.* [21,22▪▪,23▪▪] have provided excellent studies of how epigenetic mechanisms control *PRTN3* and *MPO* gene expression and their dysregulation in AAV (Fig. 1). The initial study of AAV granulocytes revealed a depletion of repressive H3K27me3 marks and an increase in mRNA expression of *PRTN3* and *MPO* [21]. In addition, a marked demethylation of a CpG island and the promoter region of *MPO* in AAV were observed, although *PRTN3* promoter region was constitutively demethylated in patients and controls. The authors then explored the regulatory mechanisms governing H3K27me3 and found enhancer of zeste homolog 2 (EZH2) interacted with Runt-related transcription factor 3 (RUNX3) to recruit H3K27 methyltransferase to *PRTN3* and *MPO*. The promoter region of the *RUNX3* gene was also hypermethylated in AAV granulocytes. This suggests a regulatory model whereby

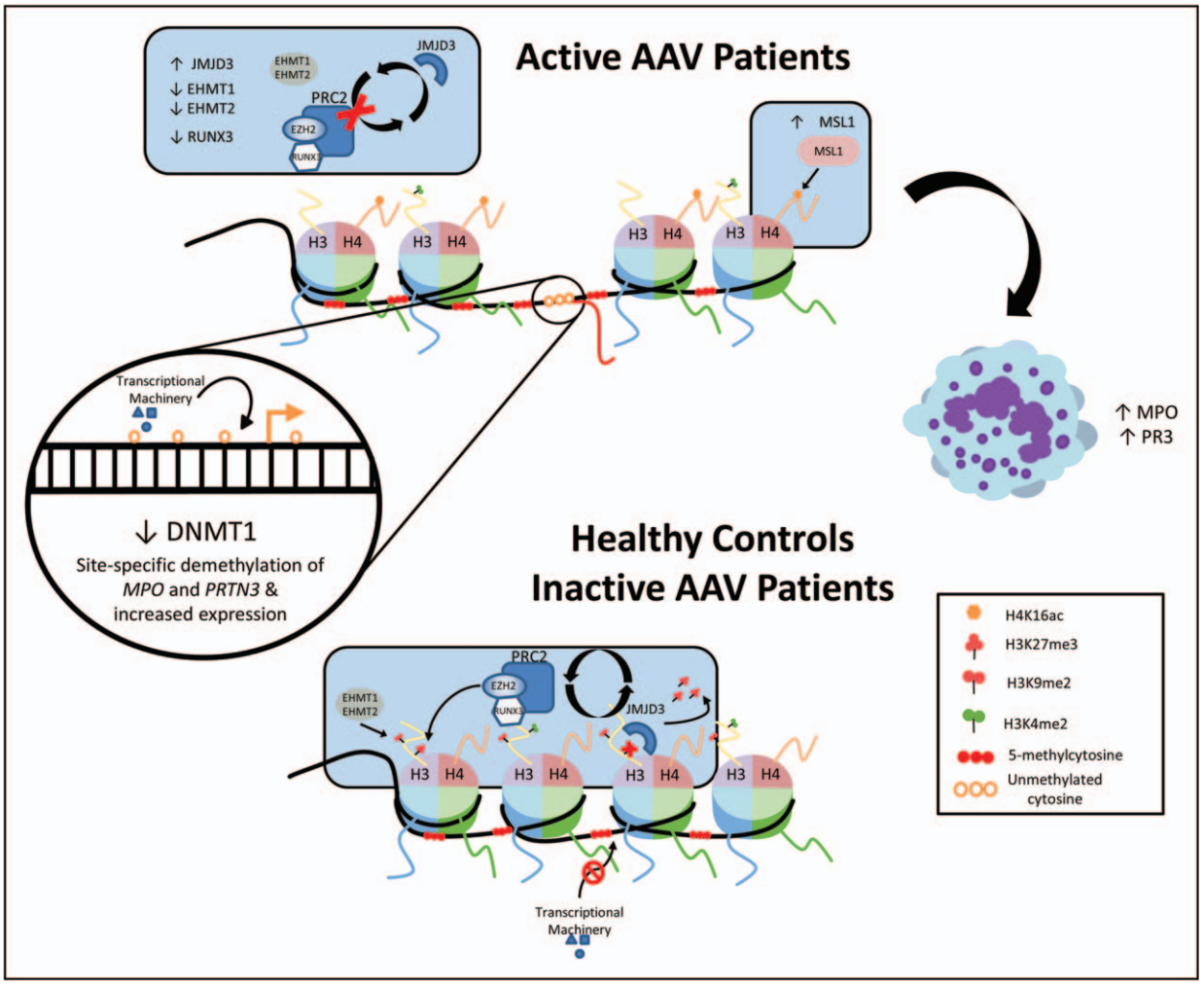

FIGURE 1. A cartoon model of epigenetic control of *MPO* and *PRTN3* in ANCA-associated vasculitis. Ciavatta *et al.* and Yang *et al.* suggest that histone modifications surrounding the promoter and enhancer regions of *MPO* and *PRTN3* in AAV are in a bivalent state (presence of both repressive and permissive marks), maintaining gene silencing in mature neutrophils that is disrupted in AAV patients. In neutrophils from healthy controls and inactive patients with low MPO and PR3 expression, JMJD3 demethylates H3K27, although PRC2 remethylates it in kind to maintain a condensed silent state. *EHMT1* and *EHMT2* assist by maintaining H3K9me2 in the same region. Permissive H3K4me2 marks suggest an epigenetic poising and are present in both patients and controls, though the *MLL2*, *MLL3*, and *MLL4* genes that regulate this mark were overexpressed in patients compared with controls. DNA methylation of the gene promoter and enhancer regions provides a second method of epigenetic control, preventing the access of transcriptional machinery, and CpG islands can be targeted by PRC2 as well for H3K27me3. In patients with active disease, some disruptive process interrupts the gene silencing and a decrease in RUNX3 expression prevents the reestablishment of H3K27me3. Decreased expression of *EHMT1* and *EHMT2* correlates with depletion of H3K9me2 and an increase in *MSL1* expression correlates with enriched H4K16ac, a mark of gene activation. Jones *et al.* found that leukocytes from active AAV patients have decreased *DNMT1* expression and a site-specific decrease in DNA methylation, suggesting a process that targets specific loci including *MPO* and *PRTN3* and allows for gene expression. When AAV is inactive, methylation at these loci is returned to levels near that of healthy controls and expression is reduced. This suggests that *MPO* and *PRTN3* DNA methylation is a disease-specific process supported by the identification of a CpG site in the *PRTN3* promoter (CpG #13) that is demethylated in patients with a higher risk of relapse. AAV, ANCA-associated vasculitis; ANCA, antineutrophil cytoplasmic antibody; MPO, myeloperoxidase; proteinase 3; PR3, proteinase 3.

hypermethylation of *RUNX3* and the loss of EZH2 and H3K27 methyltransferase recruitment is coupled with overexpression of H3K27me3 demethylase jumonji C domain-containing protein 3 (JMJD3) in AAV neutrophils. JMJD3 removes the H3K27me3 marks from regulatory regions of *MPO* and *PRTN3* and increases chromatin accessibility aided by DNA demethylation allowing access to

transcriptional machinery. Genomic regions containing genetic risk variants in AAV were found to be enriched for H3K27me3 marks that indicate a closed or poised state for the chromatin in Th17 cells, supporting the role of Th17 cells in AAV pathogenesis [24▪,25].

Yang *et al.* [23▪▪] investigated expression changes in genes encoding histone modification proteins and found a suite of four genes: euchromatic histone-lysine *N*-methyltransferase 1 and 2 (*EHMT1*, *EHMT2*) and male sex lethal 1 homolog and insulin growth factor (*MSL1* and *ING4*) with consistent expression changes in leukocytes and granulocytes from AAV patients compared with healthy controls. *EHMT1* and *EHMT2* are associated with H3K9me2, a mark of gene silencing, and were found to be underexpressed in AAV leukocytes and granulocytes. *MSL1* and *ING4* are associated with H4K16ac, a mark of gene activation, *MSL1* was found to be overexpressed in AAV leukocytes and granulocytes, although *ING4* was underexpressed in leukocytes, but not significantly underexpressed in granulocytes. These expression changes were noted to be significantly different between leukocytes from active AAV patients with elevated *MPO/PRNT3* expression and inactive patients with low-expression *MPO/PRNT3*, making them potential disease activity biomarkers. H4K16ac and H3K9me2 were, respectively, enriched and depleted at *MPO* and *PRTN3* promoter regions in AAV granulocytes, especially in more active disease. *MLL2*, *MLL3*, and *MLL4* (mixed-lineage leukemia) genes that regulate H3K4me2 were overexpressed in AAV patients compared with healthy controls. H3K4me2 is a well-recognized mark of transcriptional activation and, along with H3K27me3, is part of a bivalent chromatin signature [9▪,26]. H3K4me2 was equally enriched in both patients and controls at the *MPO* and *PRTN3* promoter region, suggesting that they remain in a poised state at these loci even in healthy cells. Taken together, *MPO* and *PRTN3* seem to maintain areas of bivalent chromatin that contain both permissive and repressive marks that are poised for expression with the loss of repressive marks. This occurs in AAV patients and is enhanced during active disease leading to abnormal over-production of granule proteins and neutrophil-mediated vascular damage.

Jones *et al.* [22▪▪] investigated DNA methylation changes in *MPO* and *PRTN3* as potential disease biomarkers in AAV total leukocytes. They noted a decrease in *DNMT1* expression but no significant reduction in global DNA methylation in AAV. However, they detected many differentially methylated genes in AAV patients, among these were *MPO* and *PRTN3* which were both hypomethylated in patients with active disease compared with healthy controls. Methylation levels in both genes were also significantly lower in AAV patients with active compared with inactive disease. In addition, DNA methylation of both *MPO* and *PRTN3* was negatively correlated with gene expression. By comparing AAV subsets (PR3-AAV versus MPO-AAV) using longitudinally collected samples from the same patients, they observed that MPO- and PR3-AAV patients experience a significant and near identical increase in the methylation level of *PRTN3* promoter after disease remission. However, only patients with MPO-AAV, and not PR3-AAV, show evidence for a significant increase in the methylation level at *MPO*, suggesting that epigenetic changes at these two loci may provide a distinction between the two disease serotypes. At both *PRTN3* and *MOP*, AAV patients who entered remission and displayed increased site-specific methylation had a significant reduction in mRNA expression of both genes, whereas those patients who experienced decreased DNA methylation upon remission displayed no change in gene expression. Perhaps the most valuable observation in this study was that demethylation of the *PRTN3* promoter region was highly predictive of disease relapse in AAV patients regardless of ANCA-serotype; patients with demethylation in *PRTN3* were 4.55 times more likely to relapse. This effect was narrowed down to a single CpG site in the promoter region of *PRTN3*. These results are very promising, but a larger study will be needed to confirm the prognostic use of this CpG site as a biomarker for relapse in AAV patients.

One drawback to this study is the use of total leukocytes in which differences in cell population proportions can mask cell-specific methylation changes from being detected. One the other hand, total leukocytes represent an easily accessible source for clinical testing and any effect that can be detected consistently in patients might have a potentially significant role in disease pathogenesis and will be valuable to develop into a disease biomarker.

GIANT-CELL ARTERITIS

Giant-cell arteritis (GCA) is an inflammatory disease of the large and medium arteries, occurring almost exclusively in people over 50 years of age, and characterized by granulomatous involvement which can lead to ischemia and necrosis or vision loss [27]. The arterial microenvironment at the site of inflammation in GCA is considerably complex with vessel-residing dendritic cells (DCs) acting as pathogen detectors and guiding T cell stimulation

and the local inflammatory response, and Th1 and Th17 cells providing proinflammatory signals [28,29]. An exploration of DNA methylation changes occurring within the temporal artery of GCA patients revealed a strong T cell-specific signature consisting of hypomethylated loci in genes involved in TCR/CD28 signaling and calcineurin (Ca)/NFAT-regulatory pathways [30▪]. NFAT is a transcription factor regulating cytokine expression in T cells, including *IFNG* and *TNF*, and *CD40LG* expression, which were also demethylated in affected arterial tissue from GCA patients. NFAT1 was also found to be localized to the nucleus of cells (suggesting dephosphorylation and activation) in the vessel wall of GCA biopsies by immunohistochemistry. Hypomethylation of cytokine genes for Th1 (*IFNG*) and Th17 (*IL6*, *IL21*) cells, macrophages (*TNF*), and DCs (*CCR7*, *CCL18*) supported their presence in immune infiltration of the vessel wall. The Ca/NFAT pathway presents an intriguing therapeutic target. Dipyridamole, a highly specific calcineurin inhibitor, is suitable for targeting NFAT-regulated expression and has been shown to inhibit the production of interferon-gamma (IFNγ), IL-17, and IL-6 in T cells from MRL/lpr lupus mouse model [31]. Many proinflammatory genes regulated by NFAT were hypomethylated in GCA-affected arteries, and there is evidence that NFAT can interact with HDAC proteins to control histone modifications in specific contexts [32].

Croci *et al.* [33▪] identified miRNAs overexpressed in GCA tissue by comparing active, nonactive, and normal artery tissue. Of these miRNAs, miR-146a, miR-155, and miR-21 were overexpressed in inflamed temporal artery tissue compared with noninflamed and normal tissue. These miRNAs play a role in the regulation of the inflammatory response in T cells, macrophages, and DCs. They are also overexpressed in abdominal aortic aneurysms and atherosclerotic plaques and might play a role in vascular remodeling [34,35]. Although none of the known protein targets of these miRNAs were differentially expressed, miR-21 expression was found to be localized to cells in the medial-intimal layer of the artery in this study.

Age itself is a considerable risk factor for GCA and is likely due in part to changes in the immune function throughout the lifespan, which is known as immunosenescence [36,37]. Age-related DNA methylation changes in CD4+ T cells suggest a pro-inflammatory epigenetic architecture with age [38]. The miRNAs highlighted by Croci *et al.* [33▪,39] have also been implicated in immunesenescence and their increased expression in GCA tissue perhaps reflects an accelerated biological age that will need to be explored further.

KAWASAKI DISEASE

Kawasaki disease (KD) is a medium-vessel vasculitis that primarily occurs in children between ages 8 months and 5 years. It is characterized by inflammation of the coronary arteries, and is the leading cause of acquired heart disease in children from developed regions [40].

DNA methylation studies of KD have revealed a relationship between *FCGR2A* methylation and response to intravenous immunoglobulin (IVIG) treatment. *FCGR2A* encodes the low-affinity immunoglobulin gamma Fc region receptor II-a protein that is expressed on the surface of macrophages, neutrophils, monocytes, and DCs, and acts to increase phagocytosis and inflammatory mediator production and contains a genetic risk variant for KD [41]. CpG sites within the promotor region of *FCGR2A* were hypomethylated in whole blood cells from KD patients compared with controls, and especially in patients resistant to IVIG treatment [42]. Another small-scale study found genome-wide, site-specific hypomethylation changes enriched in genes associated with the inflammatory immune response including *FCGR2A* [43]. This study demonstrated significant changes in DNA methylation patterns following IVIG treatment in KD, including reversal of the disease-associated hypomethylation in *FCGR2A* [43].

Toll-like receptors are a group of proteins that recognize molecular patterns, both exogenous and endogenous, and can interact with FCGR2A to induce a proinflammatory response [44,45]. A suite of TLR genes encoding TLR1, TLR2, TLR4, TLR6, TLR8, and TLR9 were found to be hypomethylated in KD patients compared with healthy and febrile controls [46]. The methylation levels of these genes were recovered within 3-week post-IVIG therapy, and mRNA expression levels maintained a negative correlation with DNA methylation.

Regulatory T cells (Tregs) play an important role in suppressing the proinflammatory activity and cytokine expression of Th17 cells through physical interactions or by releasing cytokines like IL-10 and TGF-beta. This regulatory balance seems to be skewed toward proinflammatory Th17 cells in acute KD patients where there is a reduction in FoxP3 expression, a critical transcription factor in Treg activity [47]. miR-31 expression was increased in Tregs from acute KD patients and suppresses FoxP3 expression, although miR-155, which promotes FoxP3 expression, was found to be decreased in Tregs from patients [48]. IVIG treatment partially recovered the abnormal expression of miR-31 and miR-155. Furthermore, miR-145, which might be involved in modulating TGF-beta signaling, was increased in whole blood and detected in plasma

exosomes isolated from acute KD patients [49]. Exosomes are extracellular vesicles released from cells that can be taken in by other cells and are capable of transporting miRNAs as a theorized form of cell-to-cell communication [50,51]. Other proinflammatory microRNAs that also potentially target TGF-beta signaling and are involved in KD are miR-200c and miR-371-5p. miR-200c promotes endothelial cell apoptosis, inducing vascular smooth muscle cell inflammatory response and modulating TLR4 response [52]. Both miR-200c and -371-5p were shown to effectively distinguish between KD and healthy controls as well as IVIG-responsive and nonresponsive patients [53]. More recent research has identified new miRNAs with disease activity that potentially target vessel endothelial cell functions in KD patients [54–58]. Jia *et al.* [55] performed a biomarker discovery screen on serum samples to detect exosome miRNAs in KD patients. After normalizing to internal control miRNA expression and recruiting an independent validation cohort, they identified two miRNA pairs (miR-1246/miR-4436b-5p and miR-197-3p/miR-671-5p) that when combined differentiated KD patients from healthy and febrile disease controls. These miRNA discovery studies are promising but generally relied upon small phenotypically varied cohorts of patient and controls. More work is required to validate these findings in KD, and to understand their cell-specific function and evaluate their efficacy as biomarkers.

BEHÇET'S DISEASE

Behçet's disease (BD) is a systemic, variable-vessel vasculitis of unknown cause characterized by recurrent acute inflammatory episodes with oral and genital ulcers, eye involvement, and skin involvement [59,60]. Although its pathogenesis is still currently under investigation, evidence points toward a combination of genetic and environmental triggers as contributing factors to the development of BD [59]. Genetic susceptibility for BD shows a very strong association with the *HLA-B/MICA* region, though non-MHC risk factors have been identified as well that support the involvement of Th1 and Th17 cells (*IL10*, *IL12A*, *STAT4*, and *IL23R-IL12RB2* locus) in pathogenesis [2,61–63]. This is supported by research showing that Th17, Th1, and Treg cell populations and cytokine production change with the disease state and can be found at inflammatory sites of BD patients [64–66].

DNA methylation of CD4+ T cells and monocytes extracted from the peripheral blood of BD patients are hypomethylated at genes associated with cytoskeletal remodeling processes such as actin and microtubule processing and cell adhesion [67]. Interestingly, some of the methylation deficiencies observed were returned to near those of healthy control levels at specific genes after treatment and disease remission. This recovery was more pronounced in monocytes than in T cells, but genes involved in microtubule formation and organization (*KIFA2* and *TPPP*) were affected in both cell types making them intriguing targets for clinical biomarkers and therapeutics.

Research into changes in miRNA expression in BD has revealed a variety of potential targets and biomarkers. Regulation of Th17 cell activity has shown up as a theme in miRNA research in BD. miR-23b was underexpressed in CD4+ T cells from active BD patients [68]. When transfected into CD4+ T cells *in vitro*, miR-23b reduced the expression of Notch pathway genes and production of IFNγ and IL-17 [68]. As an example of genetic–epigenetic interaction, genetic variants also play a role in miRNA functions including expression and protein targeting [69]. Two such variants have been identified in BD patients: rs2910164 (*MIR146A*; miR-146a) and rs11614913 (*MIR196A2*; miR-196a2) [70,71]. Carriers of the rs2910164 CC genotype displayed a reduction in mature miR-146a transcripts and IL-17, TNF-alpha, and IL-1 beta at the protein level in PBMCs as compared with the GG genotype which was more frequent in BD patients [70]. Carriers of the rs11614913 TT allele had reduced expression of miR-196a2 in PBMCs and the T allele was significantly more frequent among BD patients compared with healthy and disease (Vogt–Koyanagi–Harada syndrome and acute anterior uveitis associated with ankylosing spondylitis) controls and more frequent among BD patients with arthritis compared with other subgroups [71]. Reduced expression of miR-196a2 coincided with a reduction in the target protein Bach1 and an increase in proinflammatory IL-1 beta and MCP-1 cytokines [71].

IGA VASCULITIS (HENOCH–SCHÖNLEIN PURPURA)

IgA vasculitis (IgAV) primarily targets small vessels and is characterized by the deposition of IgA immune complexes in the vessel wall, and disease onset is often associated with an infection of the upper airway or gastrointestinal tract [60]. IgAV is considered a geographically and ethnically ubiquitous disease predominantly of infants and children between 3 and 12 years of age [72]. Ascertaining genetic risk is difficult because of case studies of insufficient size, but a meta-analysis confirmed the risk associated with *HLA-DRB1*01* and *HLA-DRB1*07* variants [2].

Luo *et al.* [73,74] observed a genome-wide increase in H3 acetylation and H3K4 methylation,

Table 1. Current knowledge of disease-specific epigenetic mechanisms in vasculitis

Epigenetic mechanism	Region (s) of interest	Disease-specific relationship	Function	Source	Reference
ANCA-associated vasculitis (AAV)					
Histone modification (H3K27me3)	*PRTN3*, *MPO*	Depleted H3K27me3 in AAV and corresponding increased mRNA expression; a mark of gene silencing	Neutrophil granule protein	Granulocytes	[21]
Histone modification (H3K9me2)	*PRTN3*, *MPO*	Depleted H3K9me2 at gene promoter in active AAV patients with high mRNA expression compared with healthy controls and inactive patients; regulated by EHMT1 and EHMT2 and a mark of gene silencing	Neutrophil granule protein	Neutrophils	[23▪▪]
Histone modification (H4K16ac)	*PRTN3*, *MPO*	Enriched H4K16ac at gene promoter in active AAV patients with high mRNA expression compared with healthy controls and inactive patients; regulated by ING4 and MSL1 and a mark of gene activation	Neutrophil granule protein	Neutrophils	[23▪▪]
Histone modification (H3K4me2)	*PRTN3*, *MPO*	H3K4me2 at promoter regions of both *PRTN3* and *MPO* in patients and controls; a mark of gene activation and suggests bivalent chromatin state	Neutrophil granule protein	Neutrophils	[23▪▪]
DNA methylation (promoter/CpG island)	*MPO*	Hypomethylation of promoter/CpG island of *MPO* in AAV patients	Neutrophil granule protein	Granulocytes	[21]
DNA methylation (promoter)	*RUNX3*	Hypermethylation of promoter region and reduced *RUNX3* mRNA expression in AAV patients	Recruits PRC2 that regulates H3K27 methylation of *MPO* and *PRTN3*	Granulocytes	[21]
DNA methylation (CGI/ Exon 5-6)	*MPO*	Hypomethylation of CpG island/exon 5–6 of *MPO* gene of AAV patients; typically increased with disease remission and corresponded with decreased *MPO* mRNA	Neutrophil granule protein	Total leukocytes	[22▪▪]
DNA methylation (promoter: CpG #13)	*PRTN3*	Hypomethylation of promoter of *PRTN3* gene in AAV patients; typically increased with disease remission and corresponded with decreased *PRTN3* mRNA; hypomethylation of CpG #13 is associated with increased risk of disease relapse	Neutrophil granule protein	Total leukocytes	[22▪▪]
Giant-cell arteritis (GCA)					
DNA methylation (various loci)	*PPP3CC*, *NFATC1*, *NFATC2*	Hypomethylated in GCA temporal artery; nuclear localization of NFAT1 protein in GCA-positive biopsies	Calcineurin/NFAT-signaling pathway	Temporal artery biopsy	[30▪]

Table 1 (*Continued*)

Epigenetic mechanism	Region (s) of interest	Disease-specific relationship	Function	Source	Reference
DNA methylation (cg09993145)	*RUNX3*	Hypomethylated in GCA temporal artery	Combines with T-bet for expression of IFNγ in Th1 cells	Temporal artery biopsy	[30▪,75]
DNA methylation (various loci)	*CD40LG, IL21, IL21R*	Hypomethylated in GCA temporal artery; protein present in GCA-positive biopsies	NFAT-regulated genes; involved in activation and maturation of Th cell lineages (*CD40LG*) and production of IL-17 in Th17 cells (*IL21, IL21R*)	Temporal artery biopsy	[30▪]
DNA methylation (various loci)	*CCR7, CCL18*	Hypomethylated in GCA temporal artery	Present on mature dendritic cells (*CCR7*) and attract naive T cells (*CCL18*)	Temporal artery biopsy	[30▪]
DNA methylation (various loci)	*CD3E, CD3G, CD3D, CD3Z, CD28, ZAP70*	Hypomethylated in GCA temporal artery	TCR/CD28-regulated T cell activation	Temporal artery biopsy	[30▪]
DNA methylation (various loci)	*TNF, IL2, IL1B, IL18, IFNG, LTA, LTB*	Hypomethylated in GCA temporal artery	Proinflammatory proteins and key Th1 cell cytokine (*IFNG*)	Temporal artery biopsy	[30▪]
Noncoding RNA (microRNA)	*MIR146A, MIR155, MIR21*	MicroRNAs overexpressed in inflamed GCA temporal artery	Regulation of inflammatory and vascular remodeling networks; miR-21 is localized to medial/intimal layers	Temporal artery biopsy	[33▪]
Kawasaki disease (KD)					
DNA methylation (promoter: cg24422489)	*FCGR2A*	Hypomethylated in KD patients, more so in IVIG-resistant cases; methylation increased after IVIG treatment and mRNA expression was reduced	Ig Fc receptor that regulates an array of immune responses and is a potent proinflammatory gene; genetic risk locus for KD	Whole blood	[42,43]
DNA methylation (various loci)	*TLR1, TLR2, TLR4, TLR6, TLR8, TLR9*	Hypomethylated in KD patients; methylation is restored with IVIG treatment and mRNA expression is reduced	Recognition of bacterial products and inflammatory signals	Total leukocytes	[46]
Noncoding RNA (microRNA)	*MIR145*	Increased miR-145 in whole blood of acute KD patients; found in extracellular vesicles from patient plasma	Differentiation of neutrophils and vascular smooth muscle cells and targets regulatory genes in TGF beta signaling pathway	Whole blood & plasma	[49]
Noncoding RNA (microRNA)	*MIR125A*	Increased miR-125a-5p circulating in acute and convalescent KD patient plasma	Inhibition of MKK7 expression which promotes caspase-3 expression and apoptosis in endothelial cells	Plasma	[54]

Table 1 (*Continued*)

Epigenetic mechanism	Region (s) of interest	Disease-specific relationship	Function	Source	Reference
Noncoding RNA (microRNA)	*MIR1246/MIR4436B1 & MIR197/MIR671*	MiRNA pairs that, when combined, can differentiate KD patients from controls and non-KD febrile cases	miR-197 is predictive of death in symptomatic coronary artery disease and miR-1246 is a biomarker for diastolic dysfunction	Serum exosomes	[55]
Noncoding RNA (microRNA)	*MIR155, MIR31*	Decreased miR-155 and increased miR-31 expression in CD4+CD25+Treg cells of acute KD patients; IVIG partially reverses expression difference	miRNAs expression correlated with FoxP3 mRNA in CD4+CD25+Treg cells suggesting common regulatory factors	CD4+CD25+Treg cells	[48]
Noncoding RNA (microRNA)	*MIR223*	Increased miR-223 in KD patient serum especially those with coronary artery lesions; identified as part of KD diagnostic miRNA panel in total leukocytes	Released by bone-marrow derived blood cells into serum; promotes apoptosis in endothelial cells by targeting IGF1R and suppresses cell proliferation	Serum/ total leukocytes	[56▪,57]
Noncoding RNA (microRNA)	*MIR200C, MIR371*	Circulating miR-200c and miR-371-5p are increased in KD patients; reduced after IVIG therapy	Involved in proinflammatory response	Serum	[52,53]
Noncoding RNA (microRNA)	*MIR93*	Increased miR-93 in patients responding to IVIG; inverse correlation with *VEGFA* mRNA expression	Related to expression of VEGF-A	PBMCs	[58]
Behçet's disease (BD)					
DNA methylation	*RAC1, ARHGAP24, FSCN2, BAIAP2L1, FILIP1 SSH, ANK1, MYH15, MYO1C, MYO1D, MPRIP*	Methylation changes varies with cell type, but is distinct in BD patients; methylation at some sites restored after therapy	Actin processing and cytoskeletal remodeling	CD4+T cells/ monocytes	[67]
DNA methylation	*TBCD, KIF1B, DNAH3, TUBB8, RGS14, TUBA3C, TUBA3D, TPPP, KIF2A*	Methylation changes varies with cell type, but is distinct in BD patients; methylation at some sites restored after therapy	Microtubule processing	CD4+T cells/ monocytes	[67]
Noncoding RNA (microRNA)	*MIR155*	Conflicting reports of expression changes in BD patients	Overexpression inhibits production of IL-6 and IL-1 beta and promotes IL-10 in DCs which reduced CD4+T cell expression of IL-17; targets Ets-1 which inhibits IL-17 production	PBMCs/DCs/ CD4+T cells	[76,77]
Noncoding RNA (microRNA)	*MIR146A*	Decreased miR-146a expression associated with rs2910164 (C>G) C allele which is less frequent in BD patients	Regulates proinflammatory cytokine production (IL-17, TNF-alpha, IL-1 beta)	PBMCs	[70]

Table 1 (*Continued*)

Epigenetic mechanism	Region (s) of interest	Disease-specific relationship	Function	Source	Reference
Noncoding RNA (microRNA)	*MIR196A2*	Decreased miR-196a2 expression associated with rs11614913 (C>T) T allele which is more frequent in BD patients (specifically arthritis subgroup)	Presence of variant associated with decreased miR-196a expression and an increase in target gene *BACH1*; increased production of proinflammatory cytokines IL-1 beta and MCP-1	PBMCs	[71]
Noncoding RNA (microRNA)	*MIR638*, *MIR448*, *MIR3591*	Decreased miR-638 and miR-4488 expression in stable BD patients; Increased miR-3591-3p expression in active BD patients	Regulation of IL-6 production; differential expression associated with cancers, lupus and viral infection	CD11b+PBMCs	[78]
Noncoding RNA (microRNA)	*MIR23B*	Decreased miR-23b expression in CD4+T cells of active BD patients	Regulation of Notch pathway and decreased expression of IL-17 and IFNγ in CD4+T cells	CD4+T cells	[68]
Noncoding RNA (microRNA)	*MIR139*, *MIR720*	Increased miR-139-3p and miR-720 expression in BD patients regardless of disease activity	Target Toll-like receptor and T cell receptor signaling pathways	PBMCs	[79]
IgA vasculitis (IgAV; Henoch–Schönlein purpura)					
Histone modification (H3 acetylation)	*IL4*	Increased H3 acetylation at *IL4* promoter and enhancer regions of CD4+T cells from IgAV patients	Th2 cell cytokine	CD4+T cells	[73]
Histone modification (H3K4me3)	*IL4*	Increased H3K4me3 at *IL4* promoter and enhancer regions of CD4+T cells from IgAV patients	Th2 cell cytokine	CD4+T cells	[73]
Histone modification (H3K4me)	Genome-wide	Globally increased H3K4me in PBMCs of IgAV patients with kidney disease; positive correlation with disease activity	Suggests that abnormal levels of H3K4me and maintenance proteins contributes to kidney disease in IgAV patients	PBMCs	[73]
Histone modification (H3 acetylation)	Genome-wide	Globally increased H3 acetylation in PBMCs of IgAV patients with kidney disease; positive correlation with disease activity	Suggests that abnormal levels of H3 acetylation and maintenance proteins contributes to kidney disease in IgAV patients	PBMCs	[73]

ANCA, antineutrophil cytoplasmic antibody.

which are both marks of open and transcriptionally accessible chromatin, in PBMCs isolated from IgAV patients. These marks were positively correlated with disease activity and were significantly enriched in IgAV patients with renal involvement compared with IgAV patients without renal involvement and healthy controls [73]. Coinciding with this was an increase in HATs and histone methyltransferases in IgAV patients and a decrease in the opposing histone deacetylases and histone demethylases, indicating a shift in the transcriptional profile to support an abnormal transcriptionally active state [73]. IgAV patients with renal involvement had a marked increase in IL-4, IL-6, and IL-13 at the mRNA and protein levels [73]. The authors found an enrichment of H3 acetylation and H3K4me3 at promoter and enhancer regions of *IL4*, a Th2 cytokine, in CD4+ T cells from IgAV patients compared with controls and an increased expression of TIM-1, a suggested regulator of the Th2 response [73]. By comparison, *IFNG*, encoding IFNγ cytokine of Th1 cells, displayed no enrichment for these histone marks or elevated expression [73]. An IgAV-specific global increase in open chromatin marks coupled with the open chromatin state and overexpression of Th2-related genes points toward Th2 cells as being potential effectors in the pathogenesis of IgAV. Future studies would benefit from next-generation sequencing to gain a more holistic view in which these chromatin changes are occurring in both circulating immune cells as well as those residing in the kidney, which may have different disease-specific epigenetic profiles.

CONCLUSION

Epigenetic mechanisms provide a means to understand the pathogenesis of vasculitis, improve diagnosis, monitor disease progression, and the potential identification of novel therapeutic targets (Table 1). The highly interconnected nature of these mechanisms places the emphasis of future epigenetics research in systemic vasculitis on integrating data from disease-specific and cell-specific DNA methylation states, histone modifications, and miRNA activity.

Acknowledgements

None.

Financial support and sponsorship

This work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) of the National Institutes of Health (grant number R01AR070148).

Conflicts of interest

There are no conflicts of interest.

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This study found that reduced DNMT1 expression in AAV leukocytes did not lead to global demethylation, but gene-specific hypomethylation including *MPO* and *PRTN3*. Patients who showed increased methylation with remission had an associated reduction in MPO and PR3 expression, and hypomethylation of *PRTN3* promoter region was highly predictive of disease relapse.

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Neutrophil extracellular traps in vasculitis, friend or foe?

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Purpose of review
Neutrophil extracellular traps (NETs) can be found at the sites of vascular lesions and in the circulation of patients with active small vessel vasculitis. Neutrophils from vasculitis patients release more NETs in vitro, and NETs have properties that can harm the vasculature both directly and indirectly. There are several ways to interfere with NET formation, which open for new therapeutic options. However, there are several types of NETs and different mechanisms of NET formation, and these might have different effects on inflammation. Here we review recent findings regarding the pathogenesis and therapeutic potentials of NETs in vasculitis.

Recent findings
Experimental mouse models support a role for NETs in promoting vascular damage, where histones and mitochondrial DNA appear to be driving forces. Impaired formation of NETs, however, in an SLE-like mouse model leads to more severe disease, suggesting that NETs can be important in limiting inflammation. Studies on drug-induced vasculitis reveal that levamisole can induce NETosis via muscarinic receptors, predisposing for the generation of autoantibodies, including antineutrophil cytoplasmic autoantibodies (ANCA). This supports the notion that NETs can bridge the innate and adaptive immune systems.

Summary
NETs can participate in the pathogenesis of vasculitis, but in some models there also seem to be protective effects of NETs. This complexity needs further evaluation with experimental models that are as specific as possible for human primary vasculitis.

Keywords
antineutrophil cytoplasmic autoantibodies, autoantigens, inflammation, neutrophil extracellular traps, vasculitis

INTRODUCTION

The formation of neutrophil extracellular traps (NETs) was initially described as a mechanism to ensnare and kill invading microorganisms [1], but in recent years NETs have attracted increased attention in a wide variety of medical conditions such as cancer, thromboembolism, arteriosclerosis, and autoimmune diseases [2]. Kessenbrock *et al.* [3] suggested a role for NETs in the pathogenesis of vasculitis in 2009, and since then there has been a growing body of literature on the connections between NETs and vasculitis. Here we give an overview of recent advances pertaining to altered NET formation in vasculitis, the relation between NETs and vascular damage, NETs as a source of autoantigens, the utility of biomarkers associated with NETs, and finally some possible therapeutic implications of NET formation in vasculitis.

DEFINITIONS AND NOMENCLATURE

The formation of NETs, called NETosis, was originally proposed to be a form of programmed cell death initiated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation followed by chromatin decondensation, breakdown of the nuclear membrane, and mixing of the chromatin with granule constituents [4]. The process is dependent on myeloperoxidase (MPO), neutrophil elastase, and peptidyl arginine deiminase (PAD) 4 [5,6] and results in the extrusion of a tangle of DNA decorated with citrullinated histones and other proinflammatory molecules [7]. Subsequent research, however, has questioned whether each step in this pathway is necessary for NETosis. It was, for example, recently shown that saliva can induce NETosis independently of NADPH oxidase

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Curr Opin Rheumatol 2018, 30:16–23
DOI:10.1097/BOR.0000000000000450

KEY POINTS

- There are signs of increased NETosis in tissue and blood samples from vasculitis patients.
- Neutrophils from vasculitis patients are more prone to undergo NETosis in vitro, and serum and autoantibodies from these patients induce NETosis in neutrophils from healthy controls.
- NETosis can induce endothelial damage both directly and indirectly, but NETosis may also be protective under some circumstances.
- There are several pharmacological approaches to alter NETosis, but such treatments, like the NETs themselves, might prove to be double-edged swords.

and neutrophil elastase [8]. Neutrophils are not the only cells that can extrude extracellular traps, and eosinophils, basophils, mast cells, and monocytes also have such capacity [9,10], and the term ETosis has been coined as a general term for cells releasing extracellular traps. It has been shown that extrusion of NETs is not necessarily associated with cell death, and today many authors distinguish between NETosis involving cell death (suicidal NETosis) and NETosis where the neutrophils remain viable (vital NETosis). NETs released during vital NETosis can consist of nuclear or mitochondrial DNA (mtDNA) and can be released in an NADPH-oxidase and/or reactive oxygen species (ROS)-independent manner [10–13].

Primary systemic vasculitis encompasses a wide variety of diseases with idiopathic vascular inflammation as their common defining feature. According to the current nomenclature, they are divided into groups based on the size of the vessels that are predominantly affected in the individual diseases [14]. The small-vessel vasculitides are further grouped according to immunofluorescence findings of biopsies into immune-complex vasculitides and pauci-immune vasculitides. The latter are also referred to as antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) because of their relationship to ANCA, and this group contains the diseases granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg–Strauss syndrome) [14]. More common than primary vasculitis is vasculitis as a complicating feature of other autoimmune diseases, infections, malignancies, or adverse drug reactions [15].

VASCULITIS IS ASSOCIATED WITH INCREASED FORMATION OF NEUTROPHIL EXTRACELLULAR TRAPS

The pathogenesis varies between different forms of vasculitis but at least in small-vessel vasculitis neutrophils have a prominent role. Neutrophils produce ROS and release destructive enzymes, and they attract other players to the scene through the production of cytokines and chemokines. It is often difficult to distinguish the contribution of NETs relative to activation, degranulation, and other forms of neutrophil cell death than NETosis. There are several investigations showing increased NETosis in active vasculitis, and NETs and/or remnants of NETs can be found both in the affected tissues and in the blood circulation of AAV patients [16]. Co-expression of granule proteins (such as MPO and neutrophil elastase) and chromatin (primarily citrullinated histone 3) is often considered as evidence of NETosis. One needs to be aware, however, when screening for NETs that they can also be mitochondrial-derived, and thus would not contain histones.

NETs in AAV were first reported on in kidney biopsies [3], which was later confirmed by others [17–20], and then also in skin specimens [21,22,23▪▪] and in thrombi [20,24] of these patients. Neuropathy is another common feature of vasculitis, and NETs were recently shown to be also common in nerve biopsies from AAV patients, but not seen in patients with nonvasculitic demyelinating neuropathy [25▪]. More NETs were seen in ANCA-positive MPA patients compared with ANCA-negative MPA patients and in patients with vasculitis secondary to rheumatoid arthritis [25▪]. Increased levels of breakdown fragments of NETs (NET remnants) in the circulation have also been reported in vasculitis [3,26,27▪].

In-vitro studies on neutrophils from AAV patients show that they are more prone to undergo spontaneous NETosis [18,26,28] and are more responsive to NET-inducing stimuli [29]. Similar findings have been reported in other autoimmune diseases were NETosis is implicated in the pathogenesis [30–32]. It was also recently revealed that NETosis is negatively regulated by interaction between plexin B2 on endothelial cells and semaphorin 4D on neutrophils, and NETosis in human cells can be inhibited *in vitro* by recombinant plexin B2 [33▪]. Interestingly, neutrophils from AAV patients exhibit reduced expression of semaphorin 4D compared with healthy controls [33▪], which could be an explanation for the increased amount of NETs in these patients.

SERUM, IMMUNE COMPLEXES, AND AUTOANTIBODIES FROM VASCULITIS PATIENTS INDUCE FORMATION OF NEUTROPHIL EXTRACELLULAR TRAPS IN VITRO

In-vitro studies have shown that IgG and serum from AAV patients can stimulate neutrophils from healthy controls to undergo NETosis to a greater extent than IgG and serum from healthy controls (Table 1) [3,33▪,34,35▪–39▪]. For ANCA IgG, this generally requires the neutrophils to be primed in order to increase the membrane expression of proteinase 3 (PR3) and MPO before they will respond to ANCA exposure by undergoing NETosis. This can, for example, be seen in recent reports that have shown an effect of PR3–ANCA IgG and MPO–ANCA IgG on NETosis after priming with high mobility group box 1 (HMGB1) [35▪] or tumour necrosis factor [36▪], respectively. The ability of MPO-ANCA IgG to induce NETosis appears to be related to antibody affinity rather than antibody levels [34], and it has been shown that patients with high-affinity MPO–ANCA IgG exhibit higher occurrence of NETs in renal biopsies than patients with low-affinity MPO–ANCA IgG [40▪]. However, IgG-depleted serum [38▪] and serum from ANCA-negative patients [39▪] have a similar ability as whole serum to induce NETosis, and this questions the role of ANCA IgG in these experimental settings, which did not include priming of the neutrophils before stimulation.

IgG-containing immune complexes can also induce NETosis, and the most recent study showed that these complexes activate neutrophils via cross-linking of Fc gamma receptor IIIb [41]. Another recent study found that heat-aggregated immune complexes from patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (where secondary vasculitis is common) induce NETosis, but that study did not look at receptor specificity [42▪]. IgA immune complexes in plasma and synovial fluid from rheumatoid arthritis patients were shown to induce NETosis via Fc alpha receptor I [43▪]. This most probably has a bearing on IgA vasculitis, a disease characterized by IgA immune complex deposition and small-vessel leucocytoclastic vasculitis [14]. A recent study on patients with PR3–ANCA-associated vasculitis showed that serum PR3–ANCA IgA levels were more closely related to disease activity than PR3–ANCA IgG levels [44].

Table 1. Neutrophil extracellular trap-inducing capacity of IgG and serum from vasculitis patients and healthy controls

Ab/serum	Patients	Disease activity	Priming/ activation	Patients versus HCs (+/−)	Subgroup analyses	Reference
IgG	AAV	Active and remission	Tumour necrosis factor	+	Not available	[3]
IgG	MPA	Active	Tumour necrosis factor	+	NETosis correlated with disease activity and antibody affinity	[34]
IgG	AAV	Active	HMGB1	+	Not available	[35▪]
IgG	AAV	Active	Tumour necrosis factor	+	Not available	[36▪]
Anti-lactoferrin	EGPA	Active	PMA	+	NETosis correlated with disease activity	[37▪]
Whole serum	AAV	Not available	No	+	MPA serum induced more NETosis than GPA serum	[38▪]
Whole serum	EGPA	Remission	No	+	MPO–ANCA$^+$ serum induced more NETosis than PR3–ANCA$^+$ and ANCA$^-$ serum	[39▪]
Serum from ANCA-negative patients	EGPA	Remission	No	+ (versus whole serum)	Not available	[39▪]
IgG-depleted serum	AAV	Not available	No	+ (versus whole serum)	No difference in NETosis between IgG-depleted serum and whole serum	[38▪]

+, Increased NETosis; ANCA, antineutrophil cytoplasmic autoantibody; AAV, antineutrophil cytoplasmic autoantibody-associated vasculitis; EGPA, eosinophilic granulomatosis with polyangiitis; GPA, granulomatosis with polyangiitis; HMGB1, high mobility group box 1; MPA, myeloperoxidase; PMA, phorbol 12-myristate 12-acetate; PR3, proteinase 3.

NEUTROPHIL EXTRACELLULAR TRAPS AND VASCULAR DAMAGE

There are several ways in which NETosis can harm the vasculature, both directly and indirectly. The release of noxious substances such as degrading enzymes can directly induce apoptosis in endothelial cells and degrade the basement membrane [45], and histones can be toxic to endothelial cells [46]. A recent study showed that endothelial cells can phagocytize NETs, but that excessive amount of NETs promotes vascular leakage by interfering with endothelial cell–cell interactions [47▪]. The same study also showed that NETs can induce endothelial to mesenchymal transformation (EndMT) and that such cells are increased in the glomeruli, both in MLR/lpr mice (a mouse model of SLE) as well as in patients with lupus nephritis [47▪]. EndMT is important during vascular repair but it is also connected to several disease conditions as it contributes to tissue fibrosis [48], which is a common feature in vasculitis. Indirectly, NETs promote vascular damage by activating the alternative complement pathway [49].

Renal injury is common in small vessel vasculitis, including both glomerulonephritis with crescent formation and tubulointerstitial nephritis. Recent studies have shown that NETs are present in glomeruli and that they contribute to glomerular injury in mouse models of glomerular vasculitis induced by antiglomerular basement membrane (GBM) antibodies [50▪] or GBM antiserum [46], as well as in MLR/lpr mice that spontaneously develop SLE-like disease [47▪,51▪▪]. The role of NETs in tubulointerstitial injury was shown in a study of ischemic acute kidney injury (AKI) in mice, where epithelial tubular cells during hypoxia released histones that activated neutrophils to release NETs [52▪▪]. These NETs in turn induced epithelial cell necrosis with the release of histones from these cells, thus creating a necroinflammation loop leading to enhanced tubular necrosis. This study mimics a possible scenario during excessive inflammation, with hypoxia and kidney injury, as is seen in vasculitis. Tubulointerstitial injury could also retard glomerular blood flow, thus reducing the shear stress, which has been shown to rapidly clear the glomeruli of NETotic neutrophils [50▪]. Regarding immune complex vasculitis, NETs were shown to contribute to vessel destruction and haemorrhage in mouse skin specimens after injection of bovine serum albumin (BSA) and anti-BSA antibodies [53▪].

NEUTROPHIL EXTRACELLULAR TRAPS CAN BE PROTECTIVE

A recent study showed that saliva can induce NETs, and that this capacity is diminished in Bechet's disease, a form of primary vasculitis characterized by mouth and genital ulcers [8]. The authors argued that the absence of NETs leads to diminished protection against bacteria on the mucus membranes and that this promotes ulcer formation. Other examples, in which reduced NETosis leads to more severe disease are mouse models of SLE [54▪▪] and gout [55]. These studies suggest that NETs can act as platforms to degrade proinflammatory mediators that would otherwise drive inflammation. Additionally, NETs can impair GM-CSF/IL-4-induced dendritic cell differentiation from monocytes in vitro, and can instead promote an alternatively activated macrophage phenotype [56▪]. This subgroup of macrophages is important for the resolution of inflammation, which is crucial for preventing chronic inflammation.

ANTIGEN EXPOSURE IN NEUTROPHIL EXTRACELLULAR TRAPS PROMOTES THE PRODUCTION OF AUTOANTIBODIES

NETs contain an array of molecular motifs that serve as targets for autoantibodies in autoimmune diseases, including double-stranded DNA in SLE [32], citrullinated peptides in rheumatoid arthritis [31], and MPO and PR3 in AAV [3]. NETs can also contain alarmins, such as LL39 and HMGB1 [57,58], which provide danger signals, and thus reduce immunological tolerance. The strongest evidence that NETs actually serve as a source of autoantigens driving autoantibody production in vasculitis comes from studies on drug-induced vasculitis. MPO–ANCA positivity is relatively common in patients treated with the antithyroid drug propylthiouracil (PTU), and some of these patients develop a vasculitis-like syndrome [59]. Phorbol 12-myrsetate 13-acetate (PMA) in combination with the antithyroid drug PTU induces NETs that resist DNase I degradation, and such NETs cause the production of ANCAs and AAV-like disease in rats [60]. Using a similar approach as above but in a mouse model, PMA and PTU again resulted in the production of MPO–ANCA, but did not induce disease [61▪], indicating that antibody formation is not sufficient to induce full-blown disease. Levamisole, a veterinary compound often found in adulterated cocaine, is also associated with ANCA formation and vasculitis-like syndromes. Contrary to PTU, levamisole directly induces NETosis in neutrophils in vitro via the stimulation of muscarinic receptors [23▪▪,62▪]. Also, cocaine itself is able to induce NETosis [62▪]. Patients with cocaine or levamisole-associated autoimmunity possess IgG class autoantibodies against NET components such as neutrophil elastase [62▪], PR3, MPO, LL-37, and antinuclear antibodies

[23▪▪]. Further, IgG from patients with levamisole-associated autoimmunity enhances NETosis induced by cocaine or levamisole, which could possibly create a vicious circle in these patients [62▪].

NEUTROPHIL EXTRACELLULAR TRAPS AS A BIOMARKER

Monitoring of disease activity is an unmet need in vasculitis, and better monitoring will enable more efficient use of the drugs available today and will reduce the side effects of maintenance therapy. As reviewed elsewhere [16], several studies in recent years have reported on increased levels of NETs and NET-associated proteins in the circulation of AAV patients that often correlate with disease activity. However, there is no assay available today that has proven to be clinically useful. Measurements of NETs suffer from a lack of standardisation, as well as from problems with sensitivity and specificity. As a result of this, there are currently no general values for these parameters regarding the presence of NETs in various diseases. The fact that ANCAs of different affinities appear to vary in their NET-inducing capacity encourages further studies with this approach to evaluate its usefulness to monitor disease activity [34]. The capacity for serum to degrade NETs is another tempting approach, and this capacity is reduced in serum from AAV patients [34]. DNase I activity did not vary with disease activity in that study, but NET degradation per se with serum from patients with various disease activities was not evaluated. Regardless of the methodological approach, further evaluation of NETs as a biomarker to monitor disease activity in AAV (alone or in combination with other parameters) requires carefully undertaken longitudinal studies.

IMPLICATIONS FOR TREATMENT

As summarized in Table 2, several approaches have been used in recent studies to block the effect of NETs on vascular damage in different in vivo experimental models. Antihistone treatment through antihistone antibodies, heparin, or activated protein C all proved to be efficient in treating anti-GBM-induced vasculitis [46]. This was also the case for the PAD inhibitor Cl-amidine [46], which inhibits the citrullination of histones that is an important step during suicidal NETosis. Inhibiting PAD signalling was also shown to be efficient in a mouse model of postischemic AKI [52▪▪]. Injection of adipose tissue-derived mesenchymal stem cells (MSCs) prior to injection of BSA or anti-BSA antibodies in a model of immune complex vasculitis significantly reduced the vessel damage and the amount of NETs that were formed [53▪]. The inhibitory mechanisms of the MSCs included phagocytosis of neutrophils and upregulation of superoxide dismutase 3, an antioxidant, both of which prevented NET formation [53▪]. There is a plethora of other molecules that are being tested for their ability to block NETosis, and these are primarily being evaluated in vitro. A recent example is the blockade of neutrophil elastase, which has been shown to inhibit NET-induced disruption of endothelial cell–cell integrity and EndMT [48]. Although inhibition of NADPH oxidase is effective in inhibiting suicidal NETosis in vitro,

Table 2. Therapeutic approaches that inhibit or reduce the effect of neutrophil extracellular traps on vascular damage

Therapy	Agent or approach	Experimental setup	Reference
Neutrophil clearance	AT-MSC injection, clearing via phagocytosis	Mouse model, BSA/anti-BSA, immune complex-mediated vasculitis	[53▪]
C5aR inhibitor	Avacopan	Randomized controlled trial in human AAV, phase 2	[67]
Histone neutralisation	Antihistone antibodies or heparin or activated protein C	Mouse model, sheep antirat GBM, serum-induced glomerular vasculitis	[46]
PAD inhibitor	Cl-amidine	Mouse model, sheep antirat GBM serum-induced glomerular vasculitis	[46]
PAD inhibitor	Cl-amidine	Mouse model, postischemic AKI	[52▪▪]
DNA degradation	DNase I	Mouse model, anti-GBM-induced glomerular vasculitis	[50▪]
ROS scavenging	AT-MSC injection, ROS scavenging via SOD3	Mouse model, BSA/anti-BSA immune complex-mediated vasculitis	[53▪]
mtROS inhibitor	MitoTEMPO	Mouse model, spontaneous development of SLE-like disease	[51▪▪]

AAV, antineutrophil cytoplasmic autoantibody-associated vasculitis; AKI, acute kidney injury; AT-MSC, adipocyte tissue-derived mesenchymal stem cell; BSA, bovine serum albumin; GBM, glomerular basement membrane; mtROS, mitochondrial reactive oxygen species; PAD, peptidyl arginine deiminase; SLE, systemic lupus erythematosus; SOD3, superoxide dismutase 3.

recent studies on experimental mouse models of SLE [54▪▪] and gout [55], both of which lack NADPH oxidase, show more severe diseases. In line with these studies, patients with chronic granulomatos disease, with defective NADPH oxidase, have a greater incidence of autoimmune diseases [63]. Low-density granulocytes (LDGs) are the neutrophil subpopulation that release the most NETs both in AAV [28] and SLE [32]. LDG NETs from SLE patients have been shown to be enriched for mtDNA and formation of these NETs can be inhibited in vitro with the mitochondrial ROS inhibitor MitoTEMPO [51▪▪]. Interestingly, treatment of MLR/lpr mice with MitoTEMPO limits release of mtDNA NETs and reduces disease severity [51▪▪]. Thus, targeting these NETs might also be an interesting approach in AAV. Regarding PAD4, which was shown to be a good target in various mouse models to limit NET-mediated inflammation, it was shown in the same study as for NADPH oxidase that PAD4$^{-/-}$ mice developed worse disease than control mice [54▪▪]. It appears that a certain amount of ROS signalling and NETosis is important to limit inflammation, whereas excessive NETs can instead cause disease. A recent example of compounds tested in vitro relevant to this aspect is the tetrahydroisoquinolines that can inhibit NETosis without interfering with ROS production [64▪]. These were shown to inhibit both spontaneous and PMA-induced NETosis in neutrophils from SLE patients.

DNase I, which efficiently degrades DNA and thus degrades NETs, is already being used clinically and has proven to be well tolerated. However, a phase 1 clinical study of DNase I in SLE patients did not show any effect on double-stranded DNA autoantibody production, inflammatory markers, or disease severity [65]. When applied in a mouse model of anti-GBM-induced glomerulonephritis, DNase I rescued mice from haematuria, but not proteinuria [50▪]. It appears that components of NETs such as histones and neutrophil elastase can still harm the vasculature after DNase I treatment [66]. It is worth noting that the complement fragment C5a can also induce NETosis [11], and a recent randomized controlled trial in AAV patients showed that the C5a receptor inhibitor avacopan had positive effects on disease activity [67].

CONCLUSION

Several studies have taken different approaches in studying the role of NETs in vasculitis, including in vitro functional studies, drug-induced autoimmunity, and animal models, and these have proposed NETs to constitute a source of autoantibodies and to promote vascular damage. These events can be avoided or reversed by inhibiting NETosis, blocking the proteins that are present in NETs, or by clearing NETs that have already formed. It would, however, be valuable for our understanding of NETs in vasculitis to learn more about why some animal models of autoimmune disease with impaired NETosis show aggravation of disease.

Acknowledgements

None.

Financial support and sponsorship

This review was funded by the Ingrid Asps Foundation, the Swedish Society of Nephrology, and the Swedish Renal Foundation.

Conflicts of interest

There are no conflicts of interest.

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Usefulness of PET in recognizing and managing vasculitides

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Purpose of review
The aim of this article was to review the recent contributions to the scoring methods of PET in vasculitis as well as to its role in the diagnostic work-up.

Recent findings
Both visual and semiquantitative scoring methods can be used to interpret PET scans. PET has been shown to be both sensitive and specific in the diagnosis of large-vessel vasculitis. In addition, it also has a role in predicting vascular complications.

Summary
There is a need to better standardize the scoring methods used to interpret PET scans. In clinical practice, PET is useful to diagnose untreated individuals with suspected large-vessel vasculitis and contributes to identify patients at risk for vascular complications.

Keywords
^{18}F-fluorodeoxyglucose positron emission tomography, giant cell arteritis, Takayasu arteritis, vasculitis

INTRODUCTION

^{18}F-Fluorodeoxyglucose (FDG) PET is a nuclear medicine technique, usually coregistered with computerized tomography (CT; PET/CT) or MRI; (PET/MRI), which evaluates the degree and extent of vascular uptake of the radiolabeled glucose analogue FDG by metabolically active cells in infections, malignancies and inflammation [1]. In vascular diseases, PET can demonstrate vascular inflammation both in atherosclerosis and vasculitis. In atherosclerosis, FGD vascular uptake is usually limited in extension and irregular in appearance; this 'spotty' pattern is thought to correspond to vascular areas more heavily infiltrated by macrophages (atheromatous plaques) [2] (Fig. 1). In contrast, vasculitis is characterized by a linear, smooth vascular FGD uptake extending over long vessel segments [1]. It is still debated which cells take up FDG in vascular diseases. Macrophages are believed to be involved, but there is also evidence suggesting a role for vascular smooth cells and endothelial cells [3]; activated lymphocytes are also conceivably further contributors to increased FDG uptake.

The role of PET in the diagnosis of vasculitis is mostly limited to large-vessel vasculitis (LVV), including giant cell arteritis (GCA) and Takayasu arteritis (TAK), because PET is unable to visualize vessels with a lumen diameter of 4 mm or less [4], which strongly limits its usefulness in assessing medium-vessel vasculitis. However, the diagnostic usefulness of PET is less clear in treated patients with suspected LVV, as its sensitivity declines by almost 50% shortly after institution of treatment with glucocorticoids [5].

The purpose of this article was to review recent contributions from the literature regarding the interpretation of PET findings as well as the role of PET in the diagnosis and monitoring of patients with vasculitis.

INTERPRETATION OF PET FINDINGS IN LARGE-VESSEL VASCULITIS

There is no universally accepted method to score vascular FDG uptake. A common, widely used

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Curr Opin Rheumatol 2018, 30:24–29
DOI:10.1097/BOR.0000000000000459

KEY POINTS

- PET is useful in the diagnosis of large-vessel vasculitis.
- Both visual and semiquantitative scoring methods can be used to interpret PET scans.
- The role of PET in the follow-up of patients remains unclear.
- There is a need to better standardize the methods to interpret PET scans in vasculitis.

scoring system proposed by Meller *et al.* [6] evaluates FDG uptake by a visual four-point scale, which ranges from 0 (no vascular uptake) to 1 (vascular uptake less than liver uptake), 2 (vascular uptake similar to liver uptake) and 3 (vascular uptake higher than liver uptake). In treatment-naive patients, grades 2 and 3 are considered relatively specific for active vasculitis, whereas grade 1 uptake (less commonly grade 2) can also be observed in atherosclerosis [7]. We have previously noted that involvement of arteries usually spared by atherosclerosis and a linear pattern of FGD uptake over long vascular segments further suggest vasculitis over atherosclerosis [1]; however, diffuse vascular uptake of the lower limb arteries is less specific for vasculitis [8]. On a note of caution, it should also be borne in mind that vasculitis can also be infectious in origin [9], and that FDG vascular uptake cannot discriminate per se infectious from noninfectious vasculitis. In addition, periprosthetic arterial graft uptake alone is not specific for active vasculitis [10].

Another approach to interpret vascular PET scans is to determine the maximum standardized uptake value (SUVmax) or sometimes the SUVmean of the arterial wall relative to that of a reference organ (usually the liver). There is a general consensus that vascular SUVmax is higher in vasculitis than

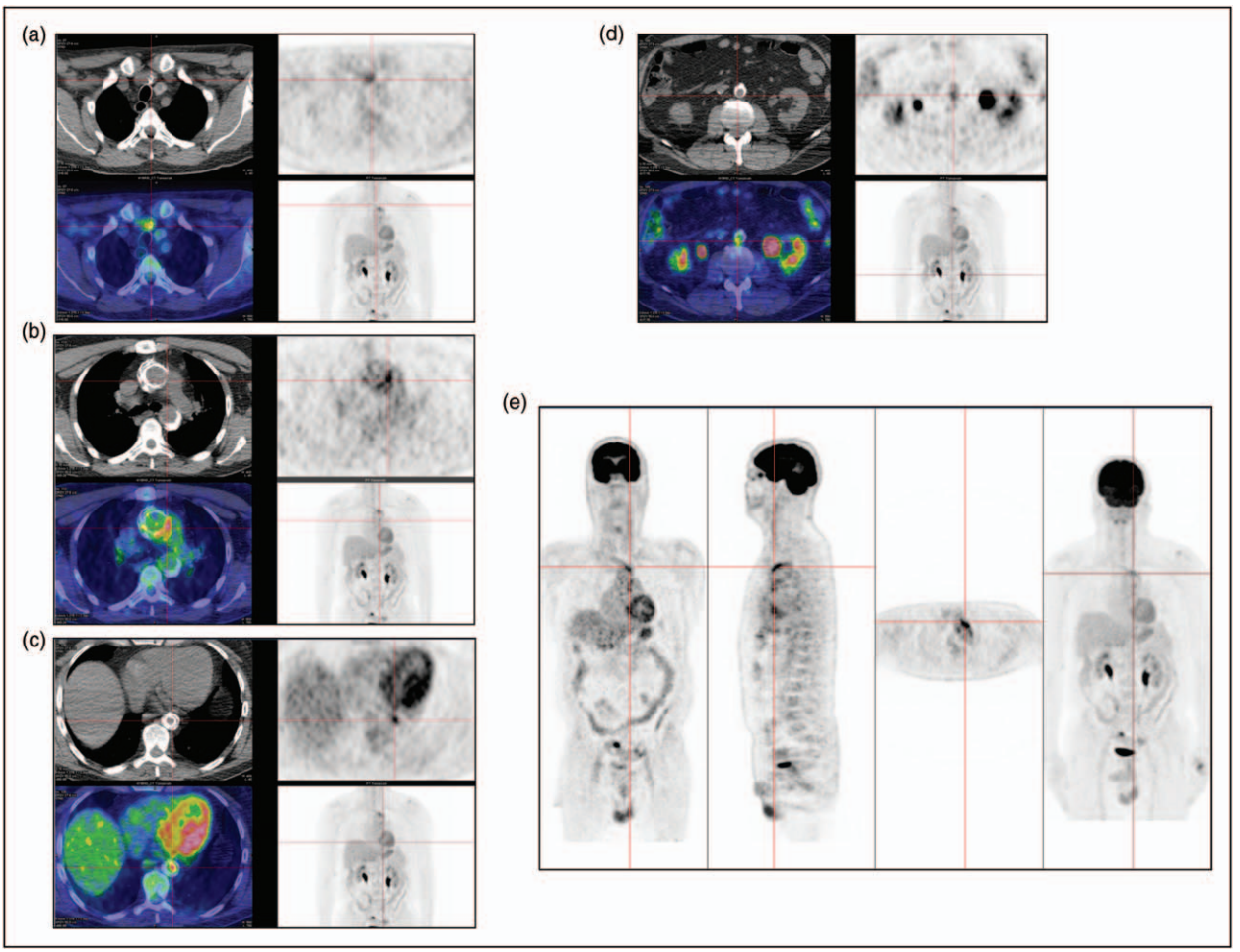

FIGURE 1. PET/CT in a subject with atherosclerosis. Typical 'spotty pattern' focal uptake of FDG in correspondence with atherosclerotic plaques appreciable in the computerized tomography imaging (correction for attenuation) at the brachio-cefalic trunk (a, transverse images; e, three axes PET images and maximum intensity projection), ascending aorta (b), thoracic aorta (c) and abdominal aorta (d).

in atherosclerosis; however, there is considerable variability in defining the optimal cutoff values that best discriminate vasculitis from atherosclerosis [11].

A comparison of visual and semiquantitative methods has recently been performed by Castellani *et al.* [12▪▪] by reviewing with both analyses 66 PET/CT images of 34 patients with LVV (six with TAK and 28 with GCA) at diagnosis and follow-up. Visual assessment was carried out using Meller's scale, whereas semiquantitative analysis was performed by calculating SUVmean in 11 regions of interest placed on the same vascular segments used for visual assessment. They found that the performance of the visual score was generally lower than that provided by semiquantitative parameters, although the difference was statistically significant only in the evaluation of the supra-aortic branches. Nevertheless, both analyses showed a very good concordance in identifying patients deemed to have active disease (87% by visual and 93% by semiquantitative scoring). The findings of this study are consistent with the results of a systematic review, which showed that visual methods were slightly less sensitive than semiquantitative ones [11]. On the other hand, visual scores were more rapid to perform and somewhat more specific than semiquantitative methods [11]. Perhaps semiquantitative analysis could be added to visual scoring in doubtful cases to increase the sensitivity of PET, but this approach remains to be validated.

ROLE OF PET IN DIAGNOSING LARGE-VESSEL VASCULITIS

Published data clearly indicate a role for PET in securing the diagnosis of LVV in treatment-naive patients. A meta-analysis showed that PET had a sensitivity of 90% and a specificity of 98% for the diagnosis of GCA, whereas it had a sensitivity of 87% and a specificity of 73% for diagnosing TAK [13].

A more recent meta-analysis investigated the diagnostic accuracy of PET in patients with a suspicion of LVV including GCA [14]. A total of eight studies involving 400 individuals (170 vasculitis patients and 230 controls) were analyzed. The pooled sensitivity and specificity of PET or PET/CT were 76% [95% confidence interval (CI) 69–82] and 93% (95% CI 89–96), respectively. When the analysis was restricted to patients with GCA, the pooled sensitivity and specificity of PET or PET/CT were 83% (95% CI 72–91) and 90% (95% CI 80–96), respectively. These results replicate the findings of the previous meta-analysis and confirm the role of PET in the work-up of patients with suspected LVV.

In selected centers, PET is currently available in coregistration with MRI. Einspieler *et al.* [15] investigated the performance of PET/MRI in patients with LVV and compared visual and semiquantitative parameters to that of PET/CT. Sixteen PET/MRI and 12 PET/CT examinations were performed in 12 patients with LVV (two TAK, 10 GCA, American College of Rheumatology criteria) and in 16 individuals matched for age, sex and risk factors for atherosclerosis. Nine patients were receiving glucocorticoids at the time of the examinations. Magnetic resonance angiography (MRA) was also carried out. Visual scores and quantitative parameters (SUVmax and target to background ratio, TBR) were compared between PET/MRI and PET/CT. TBR, SUVmax values and visual scores correlated well between PET/MRI and PET/CT ($r = 0.92$, $r = 0.91$; $r = 0.84$, respectively; $P < 0.05$). There was no significant difference between both techniques with regard to SUVmax values or visual scores. In PET/MRI, PET alone revealed abnormal FDG uptake in 86 vascular regions, whereas MRA indicated 49 vessel segments with morphological changes related to vasculitis. Overall, 40 vessel segments were concordantly positive on PET and MRI/MRA, 46 were positive on PET only and nine on MRA only. This study demonstrates that PET/MRI and PET/CT produce overall consistent results, although cannot prove whether PET/MRI is superior to PET/CT, because MRA but not CT angiography (CTA) was performed. In addition, it confirmed the added value of PET over that of MRI in disclosing affected arteries.

PET is especially valuable for diagnostic purposes in those patients who present with atypical manifestations, for example patients with GCA without cranial symptoms [16] and has been shown to increase the diagnostic accuracy over and above standard work-up examinations [5]. However, PET is less readily available and considerably more expensive than either CT or MRI. This raises the question of whether the use of PET in clinical practice is justified by an additional value over that provided by CT or MRI. The above named study by Einspieler *et al.* [15] showed that PET was able to identify more affected arteries than MRI. Another study by a French group compared the diagnostic performance of PET and CTA in 24 patients with a clinical suspicion of GCA, of whom 15 (62.5%) were ultimately diagnosed with GCA according to the American College of Rheumatology classification criteria [17▪]. Patients who had been treated with glucocorticoids for over a week were excluded. PET findings consistent with vasculitis were found in 10 patients (including three patients on glucocorticoids) who had a final diagnosis of GCA, whereas vascular FDG uptake was normal in 14 patients (five of whom were

ultimately diagnosed with GCA). Altogether, PET had a sensitivity of 67% and a specificity of 100% for the diagnosis of GCA. Regarding CTA, mural thickening suggestive of vasculitis was observed in 13 patients (11 of whom with GCA), whereas vessel wall appeared unaffected in 11 patients (seven without and four with GCA). Overall, CTA yielded a sensitivity of 73% and a specificity of 78% for the diagnosis of GCA. PET and CTA were concordant in 79% of patients; however, PET had a higher positive predictive value (100%) than CTA (85%), suggesting that it may have an edge over CTA in the diagnostic work-up of GCA.

ROLE OF PET IN PREDICTING THE COURSE OF LARGE-VESSEL VASCULITIS

As compared to its diagnostic performance, the usefulness of PET is less established with regard to predicting the course of LVV and its complications. In a study by Blockman *et al.* [18] on patients with GCA, baseline FGD uptake was shown not to correlate with the risk of subsequent relapses. On the other hand, in a study by the same group, baseline vascular FDG uptake in the thoracic aortic was only weakly associated with the risk of developing thoracic aortic aneurysms compared to patients without FGD uptake [19].

To clarify the role of PET in predicting complications of LVV, Dellavedova *et al.* [20▪▪] retrospectively reviewed PET images and clinical data of 46 consecutive patients, who had underwent PET for fever of unknown origin or suspected vasculitis before the onset of glucocorticoid therapy. The diagnosis of LVV was confirmed in 17 patients. Patients with LVV were divided into two groups, one encompassing eight patients who incurred complications (including vascular complications, relapses and at least a further positive PET scan) and one of nine patients who did not. FDG vascular uptake in the two groups was compared in terms of intensity and extension. To evaluate the extent of active disease, two volume-based parameters (commonly used in Oncology) were used: 'volume of increased uptake' (VIU) and 'total lesion glycolysis' (TLG). The threshold used to calculate VIU on vessel walls was obtained by the 'vessel to liver' ratio by means of receiver-operating characteristic analysis and was set at $0.92 \times$ liver maximum standardized uptake value in each patient. By applying this cutoff value, PET/CT was positive in all 17 patients with LVV and negative in 27 out of 29 individuals in whom LVV was excluded. Measures of vascular FDG uptake intensity (SUVmax of the vessel wall and vessel to liver ratio) were significantly greater in patients with a complicated course compared to those with a favorable one. Similarly, measures of disease extension were even more significant and TLG turned out to be the best parameter to discriminate between the two groups of patients.

The results of this study confirm the high accuracy of PET in diagnosing LVV, but also provide support to the notion that PET has a role in predicting a complicated course.

Another study that probed the capacity of PET to predict vascular complications was published last year by De Boysson *et al.* [21▪]. The specific aim of the study was to ascertain whether PET might predict subsequent aortic complications. The study cohort consisted of 130 patients with GCA who fulfilled at least three classification criteria of the American College of Rheumatology or two criteria and extratemporal histological evidence of GCA. Morphology of the aorta was assessed at diagnosis in all patients. PET/CT was performed at diagnosis in 63 (48%) patients and during the follow-up period in the 67 (52%) remaining patients. PET/CT was positive in 38 of 63 (60%) patients at diagnosis and in 31 of 67 (46%) patients during follow-up. One hundred four patients (80%) underwent at least one morphological assessment of the aorta during follow-up. Nine (9%) patients developed aortic complications (dilation in all and dissection in one) at a median time of 33 (range: 6–129) months after diagnosis. All of them had displayed large-vessel inflammation on a previous PET/CT scan. Seven patients had increased FDG uptake in the thoracic aorta and four in the abdominal aorta. All patients with increased FDG uptake in the thoracic aorta developed complications in the same vessel, whereas two out of four patients with increased abdominal aorta uptake developed abdominal aorta dilation. A positive FDG-PET/CT was significantly associated with a higher risk of aortic complications ($P = 0.004$). These findings corroborate the concept that vascular FGD uptake in an artery is a risk factor for subsequent complications in the same vessel segment, suggesting a prognostic role for PET. On the contrary, it should be kept in mind that PET per se cannot adequately visualize the vessel wall; hence, morphological imaging techniques are always required to adequately monitor patients for vascular complications over time [1].

ROLE OF PET IN MONITORING PATIENTS WITH LARGE-VESSEL VASCULITIS

Vascular FDG uptake decreases or sometimes normalizes after institution of treatment for LVV, but low-grade vascular FDG uptake may persist in some patients despite attainment of clinical remission [22,23] (Fig. 2). It is debated whether persistent

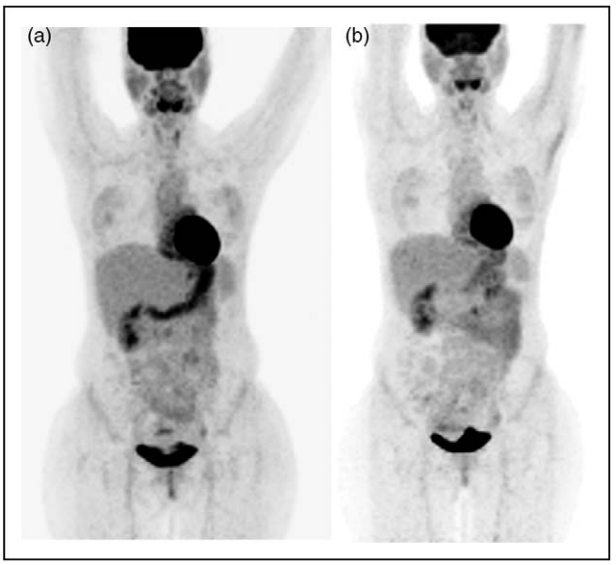

FIGURE 2. (a) (Before treatment) ^{18}F-fluorodeoxyglucose (FDG) PET of a patient with Takayasu arteritis showing grade 3 FDG uptake of the ascending tract of the thoracic aorta. (b) (After treatment): the FGD uptake of the ascending tract of the thoracic aorta has now decreased to grade 1.

low-grade vascular FDG uptake in treated patients is due to 'smoldering disease' or vascular remodeling [24]. In addition, PET findings show inconsistent correlations with inflammatory markers [12▪▪,15,20▪▪,25] and clinical indices of disease activity [12▪▪,26]. Therefore, at the present, the role of PET in the follow-up of LVV patients remains debated.

ROLE OF PET IN CHRONIC PERIAORTITIS

PET is of value in assessing chronic periaortitis, an inflammatory disorder characterized by a fibroinflammatory tissue around the retroperitoneal aorta, but also by LVV involving other arterial branches because of its capacity to visualize both the retroperitoneal mass and the affected arteries in the entire body [27,28]. With regard to the use of PET for monitoring purposes, a study demonstrated that after treatment SUVmax significantly decreased, whereas thickness of the retroperitoneal mass on MRI did not [29]. These findings suggest that PET may be more sensitive than morphological imaging in defining disease activity in chronic periaortitis, consistently with a previous report [30].

ROLE OF PET IN THE ASSESSMENT OF MEDIUM-VESSEL VASCULITIS

PET is not routinely used to evaluate patients with medium-vessel vasculitis because the caliber of the vessels involved is below its power of resolution [4]. A review of the role of PET in ANCA-associated vasculitis (AAV) revealed that PET is mostly unable to detect sites of active disease over and above those found during standard work-up; in addition, PET usually cannot identify skin, joint, eye and peripheral nerve involvement [31]. In a patient with eosinophilic granulomatosis with polyangiitis, PET proved less sensitive than chest CT in detecting pulmonary lesions; further, it failed to capture otitis media and sinusitis [32]. Finally, in the setting of AAV, PET cannot differentiate inflammatory from neoplastic lesions [33]. In polyarteritis nodosa, a disease often characterized by 'patchy' muscle involvement on MRI [34], FDG subcutaneous and muscle uptake in a patient was likewise patchy, producing a 'leopard skin' appearance on PET [35]. As a rule, however, PET is not indicated in patients with a suspicion of small or medium-vessel vasculitis.

CONCLUSION

PET is useful in recognizing LVV, but its scoring methods need standardization.

Acknowledgements

The authors wish to acknowledge Dr Massimo Casali, Nuclear Medicine, Reggio Emilia Hospital, for kindly providing us with the PET images.

Financial support and sponsorship

N.A.M.P.: Guest speaker at UCB-sponsored meetings: (Immunology Summits, Prague, 2012, 2013 and 2014, MACRO Meet the expert at the ACademy of RheumatOlogy, Bologna 13–14 April 2012, GRAPPA Workshop, Milan 29 January 2016) and Alfa-Wassermann sponsored meeting (Rhewind, Bologna, February 2016). Received royalties from Uptodate.com. PI for Italy for the gevokizumab in myositis Servier study (2014) and the sirukumab in GCA GSK study (2016). A.V.: none. C.S. received consulting and investigator fees from Abbvie, Pfizer, MSD, Roche, Celgene and Novartis.

Conflicts of interest

There are no conflicts of interest.

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Biomarkers in vasculitis

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Purpose of review
Biomarkers are considered to be helpful in diagnosing, monitoring, predicting treatment response, and prognosis in clinical practice and as outcomes in clinical trials. In this article, we review the recent literature on new biomarkers and the expanding use of older ones in vasculitic conditions.

Recent findings
In antineutrophil cytoplasmic antibody-associated vasculitis patients antineutrophil cytoplasmic antibody type may be useful as a predictor of relapse and response to rituximab. Moreover, serial measurements of proteinase-3 titer may help to predict relapse. Urinary soluble CD163 levels are promising for identifying active renal vasculitis. Imaging modalities such as positron emission tomography, computerized angiography tomography, and temporal artery ultrasound maintain their role in diagnosis and disease assessment in large vessel vasculitis. Fecal calprotectin is a useful marker of active gastrointestinal involvement in Behçet's syndrome.

Summary
The publications reviewed here potentially may help to move the field of biomarkers in vasculitis management. However, more work toward understanding the underlying pathophysiology and effects of an intervention on the disease process are needed before true biomarkers can be realized. Further studies with appropriate control groups, using good definitions for disease states such as activity and remission are needed to guide our use of these markers correctly in the management of our patients.

Keywords
antineutrophil cytoplasmic antibody-associated vasculitis, Behçet's syndrome, biomarker, Kawasaki disease, large vessel vasculitis

INTRODUCTION

The National Institutes of Health Biomarkers Definitions Working Group defined in 1998 a biomarker as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' [1]. This was later expanded to include 'any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease' [1]. Biomarkers are not only commonly used in clinical care but are even more commonly used as primary outcomes in clinical trials, along with clinical signs and symptoms as endpoints. However the main issue is to determine the relationship between any biomarker and relevant clinical endpoints. Biomarkers can serve to replace clinical endpoints only when the underlying pathophysiology is clearly understood and predictive power of the biomarker is relevant and useful in either diagnosis, treatment, or prognosis not only of groups of patients but also at the individual patient level. In rheumatology, especially in the area of vasculitis, we are rather far from this desired state of affairs. In this article we review the recent literature in this effort to identify new biomarkers and the expanding use of older ones in vasculitic conditions.

ANTINEUTROPHIL CYTOPLASMIC ANTIBODY-ASSOCIATED VASCULITIS

Currently used biomarkers in antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) are inflammatory markers including erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), and ANCAs [2]. Among these, ANCAs are considered to be most promising. Several groups

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Curr Opin Rheumatol 2018, 30:30–35
DOI:10.1097/BOR.0000000000000447

KEY POINTS

- ANCA type and increase in proteinase-3-ANCA titer during follow-up may help to predict relapses in AAV patients and proteinase-3-ANCA may also be associated with better RTX.
- Urinary sCD163 levels may be useful for identifying active renal vasculitis, whereas hypocomplementemia may be associated with poorer overall and renal survival in ANCA-associated vasculitis.
- BVAS and FFS at baseline may be associated with relapse rates and mortality in PAN.
- Imaging modalities such as PET, computed tomography angiography, MRA, and temporal artery ultrasound remain to be important tools for diagnosis and follow-up of patients with GCA and Takayasu arteritis, whereas further work is needed to identify serum biomarkers.
- Fecal calprotectin seems to be a useful marker of active gastrointestinal involvement in Behçet's syndrome.

looked at whether the titer is associated with disease activity or severity and whether ANCA type is more important in determining drug response and disease prognosis than clinical diagnosis. This year, four interesting studies that explored the role of ANCA type were published. When the AAV patients in the Rituximab for ANCA-associated Vasculitis (RAVE) trial were grouped by clinical diagnosis or ANCA type, the proportion of patients achieving complete remission at month 6 were similar among proteinase-3-ANCA vs. myeloperoxidase (MPO)-ANCA and granulomatosis with polyangiitis (GPA) vs. microscopic polyangiitis (MPA) [3▪]. However, response to rituximab (RTX) was significantly higher among proteinase-3-ANCA group, whereas there was no difference between GPA and MPA groups. Murosaki *et al.* [4] reported a higher relapse rate in proteinase-3-ANCA group but no difference regarding overall and renal survival between proteinase-3-ANCA and MPO-ANCA groups. Schirmer *et al.* [5▪] divided AAV patients into three groups: proteinase-3-ANCA positive GPA, MPO-ANCA positive GPA, and MPO-ANCA positive MPA. Mortality was significantly higher in MPO-ANCA positive MPA group than proteinase-3-ANCA positive GPA group. When patients were stratified according to their diagnosis (GPA vs. MPA), mortality was higher in the MPA group. However, there was no difference regarding survival when they were analyzed by the ANCA type. Relapse rates were not different among the three groups and GPA vs. MPO, whereas an increased relapse rate was observed among patients with proteinase-3-ANCA irrespective of diagnosis. Miloslavsky *et al.* [6▪] reported that MPO-ANCA positive GPA had a higher relapse rate than MPO-ANCA positive MPA, whereas the relapse rates were similar among MPO-ANCA positive GPA vs. proteinase-3-ANCA positive GPA and proteinase-3-ANCA positive GPA vs. ANCA negative GPA. In summary, these findings suggest that ANCA type may be more important in predicting relapse than the clinical diagnosis of GPA or MPA.

The utility of serial ANCA titer measurement to predict relapse have been studied in the RAVE trial and an increase in proteinase-3 levels was found to predict severe relapses among patients with renal involvement or alveolar hemorrhage or those treated with RTX [7▪▪]. Increased serum calprotectin levels at month 2 and 6 compared with baseline were associated with earlier and more frequent relapses among proteinase-3-ANCA positive AAV patients treated with RTX [8].

Urinary soluble CD163 (sCD163) was proposed as a biomarker for active renal vasculitis as macrophages are the most common inflammatory cells in glomerular crescents [9▪]. A cutoff value of 0.3 ng/mmol distinguished active renal vasculitis from inactive vasculitis and extrarenal active vasculitis, with a sensitivity of 83% and a specificity of 96%. However, the elevated urinary sCD163 levels in the subgroup of diseased controls may limit its use. Serum sCD163 levels failed to identify disease activity and distinguish infections from active disease [10].

The role of the complement system in the pathogenesis of AAV has been supported in a phase 2 randomized controlled study showing the efficiency of avacopan (selective complement component 5a receptor inhibitor) in AAV patients [11]. Over the last year, three studies evaluated the prognostic implications of hypocomplementemia in AAV patients. Low serum C3 levels at disease onset were associated with poorer overall and renal survival in two studies [12,13]. However, all patients in the study of Augusto *et al.* [12] and 8% of the patients in the study of Villacorta *et al.* [13] had normal serum C3 levels. The third study defined patients with hypocomplementemia as having low serum C3 and/or serum C4 and/or total complement activity. Patients with hypocomplementemia had higher mortality, but no difference regarding renal survival. Finally, a histopathology study showed that complement component 3d-positive glomerular staining is associated with the development of end-stage renal disease [14]. However, none of the studies provided a cutoff value for clinical use. These studies supported the role of the alternative rather than the classic complement pathway in AAV, considering that there was no relationship between sC4 levels and C4d glomerular staining and prognosis.

Four recent studies reexamined the predictive value of the renal histopathologic classification by Berden *et al.* [15] (focal, crescentic, mixed, and sclerotic with an ascending sequence) on renal outcome. These studies suggested that: sclerotic class was more prone to relapse probably because of the lack of compensatory ability of the sclerotic kidneys [16], patients without interstitial inflammatory infiltrates carry a higher relapse risk [16], no difference in the development of end-stage renal disease among mixed vs. crescentic classes [17,18], the crescentic class may be subdivided according to the percentage of extracapillary proliferation as the prognosis of crescentic class with more than 75% crescents was as bad as that of the sclerotic class [19].

Formation of neutrophil extracellular traps (NETosis) describes the extracellular structures formed by neutrophils to eliminate microbes. However, it may promote autoimmunity by inducing posttranslational modification of host proteins [20]. The role of NETosis in the pathogenesis of AAV was first described in 2009 [21], and NETosis was found to be present in glomerular and skin lesions and thrombus formation in patients with AAV [22]. During the last year, one study from Poland found a correlation between disease activity and NETosis in patients with proteinase-3-ANCA [23], whereas another study from China demonstrated that it was not useful in disease activity assessment in patients with MPO-ANCA [24]. This difference may be related to the different methodology used for measuring NETosis, and the inclusion of different patient populations.

POLYARTERITIS NODOSA

Over the last year, Oh *et al.* [25] from South Korea, investigated whether clinical and laboratory data, Birmingham vasculitis activity score (BVAS) and five factor scores (FFS) at diagnosis could predict relapse in polyarteritis nodosa (PAN). The mean initial BVAS and FFS 1996 of patients in relapse group were higher than those of patients in no relapse group ($P < 0.005$ for both). Patients having initial BVAS over 13.5 and FFS 1996 over one exhibited significantly higher risk of relapse [relative risk (RR) 40.0 and RR 7.0, respectively]. However, only initial BVAS over 13.5 remained significant in Kaplan–Meier survival analysis and the authors concluded that BVAS over 13.5 at diagnosis was the only independent predictor of relapse of PAN.

In a similar study, Abe *et al.* [26] reviewed predictive factors for mortality related to PAN among Japanese patients. In this retrospective single center study, 18 patients who died because of PAN were examined. Baseline 2009 FFS was associated with mortality, with a hazard ratio of 2.34 ($P = 0.04$). Analysis of relapse-free survival time showed an association with rapid progressive renal failure, BVAS, the 1996 FFS, and the 2009 FFS, with hazard ratios of 7.28 ($P = 0.048$), 1.26 ($P = 0.02$), 2.32 ($P = 0.03$), and 1.82 ($P = .04$), respectively. The authors concluded that BVAS and the 1996 FFS at diagnosis may be prognostic factors for relapse-free survival, and the 2009 FFS at diagnosis may be a prognostic factor for both mortality and relapse-free survival and these may be useful tools in the everyday management of these patients.

KAWASAKI DISEASE

This year, studies on Kawasaki disease have mainly focused on the predictors of intravenous immunoglobulin responsiveness. Combined use of neutrophil-to-lymphocyte and platelet-to-lymphocyte ratios [27], QT interval dispersion [28], serum prostaglandin E2 levels [29], the ratio of $CD8^+$ human leukocyte antigen-DR and T cells/$CD8^+$ $CD69^+$ T cells [30], and serum tenascin-C levels [31] were evaluated for the first time and were proposed as useful biomarkers for predicting intravenous immunoglobulin resistance in patients with Kawasaki disease. However, these biomarkers need to be further validated.

A meta-analysis has shown that N-terminal probrain natriuretic peptide may be a useful diagnostic test [32]. For the first time, Kwon *et al.* studied its diagnostic performance in hyperacute phase of Kawasaki disease and showed that it may be an adjunctive diagnostic test in patients with less than 5 days of fever [33]. Additionally, CD64 expression [34] and a urine-based diagnosis by colorimetric sensor array [35] were reported as candidate diagnostic markers.

LARGE VESSEL VASCULITIS

Imaging modalities, genetic markers, serum, and tissue biomarkers have been studied for their potential to diagnose, identify active or severe disease, and predict drug response in giant-cell arteritis (GCA) and Takayasu arteritis. However, none of these markers have been proven to have enough reliability to be currently used in patient care. ESR and serum CRP levels are routinely checked, while their potential false positive and false negative results for showing disease activity are well recognized.

For both Takayasu arteritis and GCA, imaging has been studied extensively as a diagnostic marker. A large multicenter study temporal artery biopsy versus ultrasound for the diagnosis of giant cell arteritis (TABUL) suggested a higher sensitivity (54 vs. 39%), but lower specificity (81 vs. 100%) for ultrasound

compared to temporal artery biopsies in diagnosing GCA [36▪]. A recent meta-analysis of the diagnostic accuracy of 18F-fluorodeoxyglucose (FDG)-positron emission tomography (PET) or PET/computed tomography (CT) scans showed a pooled sensitivity of 75.9% (95% confidence interval 68.7–82.1) and specificity of 93.0% (95% confidence interval 88.9–96.0) [37]. Two studies comparing the performance of CT angiography with FDG-PET showed that both have a high diagnostic yield for GCA with a somewhat better performance of FDG-PET to detect inflammation [38▪,39▪]. Contrast enhanced magnetic resonance angiography (MRA) had a positive predictive value of 92% and negative predictive value of 88% when temporal artery biopsies were used as the gold standard [40]. However, the probability of positive MRA after 5 days of glucocorticoid treatment was reduced by 89.3%.

There were few studies that elaborated on imaging as a marker of disease activity and progression. A multicenter cohort study showed that a positive PET scan was a good predictor of future aortic complications such as dilation and dissection in GCA [41▪]. Contrast enhanced ultrasonography vascularization grade in the carotid arteries showed good correlation with vascular inflammation on ^{18}F-FDG-PET in patients with GCA and Takayasu arteritis [42]. Both modalities showed positive results more frequently in active disease according to Kerr's criteria in both Takayasu arteritis and GCA patients [42]. A subgroup analysis of the TABUL study suggested that the halo sign on temporal artery ultrasound decreased rapidly with corticosteroids and correlated with the presence of ischemic symptoms, suggesting potential use as a diagnostic and prognostic marker (A. S. Serafim, unpublished data).

Serum cytokines were studied as potential biomarkers for disease activity in Takayasu arteritis with controversial results. A recent study showed elevated serum interleukin (IL)-6 and soluble IL-6 receptor levels in both Takayasu arteritis and GCA patients compared with healthy controls [43]. Soluble IL-6 receptor levels correlated with disease activity, while IL-6 levels were not in Takayasu arteritis and the reverse was true for GCA [43]. A study in Indian Takayasu arteritis patients showed that interferon-α levels, but not IL-6, IL-17, IL-23, IL-10, or transforming growth factor-β were higher in active patients compared with stable patients and were correlated with ESR [44]. None were associated with the drug response [44]. In a study among Japanese Takayasu arteritis patients, tumor necrosis factor-α and IL-6 levels were higher in patients with active disease, were correlated with ESR and CRP levels, and decreased with drug response [45]. However, these were not true for IL-12 or IL-23 levels [45]. The choice of controls and the difficulty in defining active disease seems to be the source of bias in these studies. The clinical activity scores, ESR and CRP levels, and PET scans that are frequently used as the gold standards are far from being perfect as predictors of disease activity. Moreover, controls with infections and longitudinal studies are mandatory to understand the real value of these biomarkers in clinical practice.

Previous studies showed that pentraxin-3 was correlated with indicators of vascular inflammation such as angiography findings, vascular wall enhancement on MRA after contrast media infusion, and positive staining in endothelial vaso vasorum cells of surgically resected aortic aneurysms, but not with systemic inflammation markers such as ESR and CRP rendering pentraxin-3 a potentially important biomarker for Takayasu arteritis [46–48]. A recent study suggested that pentraxin-3 does not seem to be a useful biomarker as pentraxin-3 levels were similar in active and inactive Takayasu arteritis patients according to physicians global assessment, the Indian Takayasu Clinical Activity Score 2010, and Kerr's criteria despite being correlated with CRP levels [49]. High levels of plasma pentraxin-3 were also previously reported in GCA patients [50]. Other recently studied potential biomarkers were soluble human leukocyte antigen-E for Takayasu arteritis [51] and vascular endothelial growth factor for GCA [52].

BEHÇET'S SYNDROME

The diagnosis of Behçet's syndrome relies mainly on a clinical assessment of current and past findings. A metabolomic profiling study using gas chromatography with time-of-flight mass spectrometry to identify potential biomarkers for the diagnosis of Behçet's syndrome showed that a panel of five metabolic biomarkers, decanoic acid, fructose, tagatose, linoleic acid, and oleic acid had a sensitivity of 100% and specificity of 97.1% for diagnosing Behçet's syndrome [53].

Four studies tested several cytokines as potential markers of disease activity for Behçet's syndrome over the last year [54–57]. Although many cytokines showed elevated levels in Behçet's syndrome patients compared with healthy controls, only IL-2 was associated with disease activity when tested among Behçet's syndrome patients with uveitis [54]. A recent study that explored several autoantibodies as potential biomarkers for Behçet's syndrome showed that only antilysozyme levels were correlated with disease activity [58].

Serum amyloid A level was significantly higher in Behçet's syndrome patients with gastrointestinal

involvement compared with the healthy controls. However, it was not correlated with disease activity, whereas serum CRP level was [59]. Fecal calprotectin was significantly higher among Behçet's syndrome patients with intestinal ulcers compared with those with normal colonoscopies, and its level was correlated with the disease activity index for intestinal Behçet's syndrome [60▪]. Moreover, significantly higher levels were reported in Behçet's syndrome patients with active gastrointestinal involvement compared with Behçet's syndrome patients who had gastrointestinal involvement, but were in remission at the time of stool collection (S.N.E., unpublished data).

CONCLUSION

Biomarkers play an important role in improving the diagnosis, treatment, and prognosis of patients with vasculitic conditions. They are also potentially useful in medical research and drug development. Biomarkers should only be considered as replacements for clinical relevant findings and endpoints if we understand the normal physiology of a biological process, the pathophysiology of that process in the disease state, and effects of an intervention – pharmacological, device, or otherwise – on these processes. While publications we have reviewed help move the field of biomarkers in vasculitis management in this direction, there is still a lot of work that needs to be done.

Acknowledgements

None

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

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Accelerated atheromatosis and arteriosclerosis in primary systemic vasculitides: current evidence and future perspectives

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Purpose of review
Primary systemic vasculitides (PSV) encompass a subset of autoimmune diseases, characterized by inflammation of blood vessels. Atheromatosis and arteriosclerosis may be accelerated in several PSV and account for the increased rate of cardiovascular morbidity that some exhibit. We aimed to summarize recent studies reporting on the acceleration of atheromatosis and/or arteriosclerosis in each type of PSV, using state-of-the-art noninvasive vascular biomarkers with clinical value as end points.

Recent findings
Limited number of PSV patients and methodology limitations reduce the value of many published studies. Accelerated atheromatosis, as measured by the use of carotid ultrasonagraphy (plaques and intimal–medial thickening) and increased arterial stiffening, as measured by the use of applanation tonometry (carotid to femoral pulse wave velocity), are currenly well established in Takayasu arteritis, Kawasaki disease and Behcet's disease. The association of atheromatosis and arteriosclerosis with polyarteritis nodosa and small vessel vasculitides remains less established and studied, so far.

Summary
Accelerated atheromatosis and arteriosclerosis or arteriosclerosis are established in some PSV. The potential clinical value of easy-to-measure and clinically useful noninvasive vascular biomarkes prompts the need for large prospective cohorts in order to provide useful future guidance regarding the prognosis and treatment of PSV patients.

Keywords
arteriosclerosis, atheromatosis, intima–media thickness, primary vasculitides, pulse wave velocity

INTRODUCTION

Primary systemic vasculitides (PSV) is a heterogeneous group of rare and potentially life-threatening diseases characterized by inflammation of the vascular wall [1▪,2]. The size and localization [4] of the involved vessels in association with the nature of the inflammatory process (focal or systemic, presence of necrosis, immune complex formation) account for the variability of the clinical manifestations between the various PSV [2]. Prior to the introduction of corticosteroids, the natural history of untreated PSV was that of a rapidly progressive and usually fatal disease [3,4]. Nowadays, the causes of death include cancer and infections because of chronic immune activation and/or immunosuppressive therapy [4,6]. Premature deaths may also occur because of acute renal failure and pulmonary hemorrhage, especially in small vessel vasculitides [5], whereas macrovascular complications (e.g. coronary artery disease, stroke, aneurysm formation and rupture) are the leading causes in medium and large vessel vasculitides [3,4,5]. Vascular damage in PSV is primarily characterized by lumen stenosis, occlusion or aneurysmal dilatation of blood vessels because of intramural inflammation and necrosis [3]. Mural fibrin deposition in arterioles or venules as well as angiocentric inflammatory cell infiltration are the hallmarks of biopsy-proven

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Curr Opin Rheumatol 2018, 30:36–43
DOI:10.1097/BOR.0000000000000453

KEY POINTS

- The limited number of primary systemic vasculitides (PSV) patients and methodology limitations (lack of prospective data, often misuse the interpretation of vascular biomarkers) reduces the value of many published studies.
- Strong data on the acceleration of atheromatosis and increased arterial stiffening are currently present in Takayasu arteritis, Kawasaki disease and Behcet's disease.
- The association of atheromatosis and arteriosclerosis with polyarteritis nodosa and small vessel vasculitides remains the least established, so far.
- The potential clinical value of noninvasive vascular biomarkers such as carotid intimal–medial thickening and carotid to femoral pulse wave velocity prompts the need for large prospective cohorts in order to provide useful future guidance regarding the prognosis and treatment of PSV patients.

diagnosis [3]. The early steps in the immunological process of vascular damage in PSV cannot be considered to be uniform, as various discrete mechanisms such as endothelial activation and dysfunction, autoantibodies to endothelial cell-surface antigens or neutrophil components and abnormal IgA tissue deposition are involved [5]. Moreover, emerging evidence suggest that the two classical pathways of arterial damage, namely, atheromatosis (i.e. atheromatic plaque formation), and arteriosclerosis (i.e. arterial stiffening), are accelerated, thus participating in the development of microvascular and macrovascular complications in PSV [4,7▪▪,8]. Herein, we aim to summarize recent studies reporting on accelerated atheromatosis and arteriosclerosis or arteriosclerosis in each type of PSV. To this end, we also present the major, widely applied noninvasive vascular biomarkers used to assess atheromatosis [intima–media thickness (IMT), plaque presence] and arteriosclerosis [pulse wave velocity (PWV) and carotid disentibility] in clinical research and practice. Secondary vasculitides have been excluded from the present review, as these entities involve additional pathological mechanisms.

POTENTIAL MECHANISMS OF ACCELERATED ATHEROMATOSIS AND ARTERIOSCLEROSIS IN PRIMARY SYSTEMIC VASCULITIDES

Although it is widely accepted that atherosclerosis involves an ongoing inflammatory response [9], the potential mechanisms of this phenomenon are yet poorly studied in PSV. Although major differences do exist, one might consider that the acceleration of arterial damage in PSV shares several common mechanisms with rheumatoid arthritis (RA), which is by far, the most extensively studied model of arterial damage in chronic inflammatory diseases [10,11]. Therefore, cautious extrapolation of these mechanisms to PSV seems reasonable. In brief, these mechanisms involve: the primary intramural vessel wall inflammation (Box 1, Fig. 1); the secondary vessel wall inflammation by systemic inflammation (Box 2. Figure 1); PSV-related drug treatment-induced deleterious effects on the vessel wall

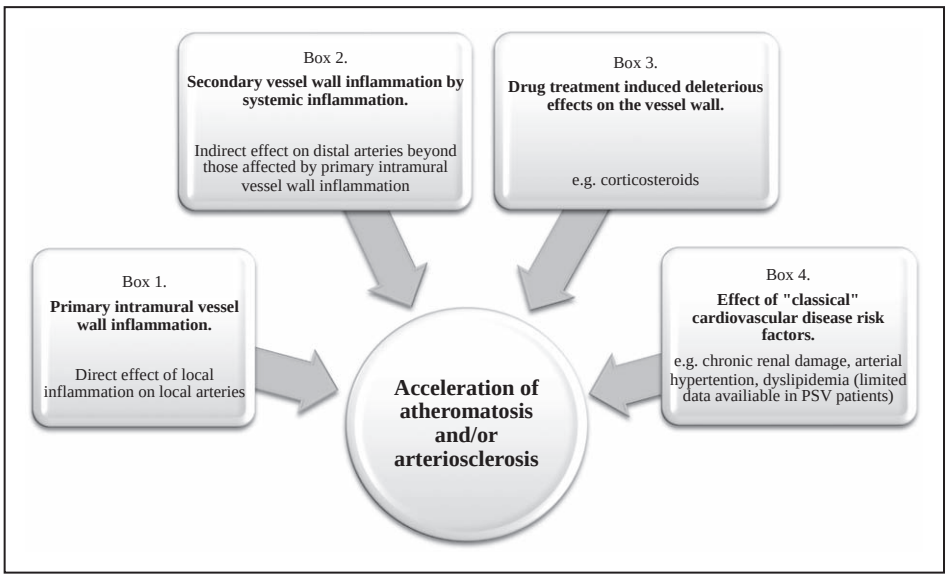

FIGURE 1. Potential mechanisms leading to the acceleration of atheromatosis and arteriosclerosis in primary systemic vasculitides (PSV).

(Box 3, Fig. 1) and the effect of classical cardiovascular disease risk factors (Box 4, Fig. 1) [7▪▪].

Data on the prevalence of hypertension and dyslipidemia are currently lacking in PSV patients, but an increased incidence compared with the general population is thought to result from the chronic use of corticosteroids, the often presence of chronic renal damage, as well as the chronic inflammatory process per se. These general mechanisms may not only precipitate the classical inflammatory process of atheromatosis [7▪▪], but also may account for the disruption of the balance between synthesis and degradation of collagen and elastin, leading to vascular stiffening [8].

NON-INVASIVE VASCULAR BIOMARKERS FOR THE ASSESSMENT OF ATHEROMATOSIS AND ARTERIOSCLEROSIS

Atheromatosis (atheromatic plaque formation) and arteriosclerosis (reduced elasity because of elastin fiber loss or dysfunction) represent two distinct pathways of arterial damage, which although share some common risk factors but have different damage and pathophysiological consequences. A large variety of noninvasively assessed vascular biomarkers have been developed during the past 30 years to describe these two pathways [12▪▪]. The most widely applied and herein used biomarkers are described in Table 1. These biomarkers are used to investigate the damage of the artery and optimize cardiovascular risk stratification in clinical practice but currently cannot be used in treatment follow-up [12▪▪].

LARGE VESSEL VASCULITIDES

Takayasu arteritis

Takayasu arteritis is a chronic inflammatory granulomatous vasculitis, manifesting mainly as a panaortitis and occuring commonly in young women between 10 and 30 years [13]. Takayasu arteritis is associated with a significantly increased risk of cardiovascular complications, including cerebrovascular events, aortic aneurysm formation and ruptured and congestive heart failure. The mechanisms that link Takayasu arteritis with late cardiovascular complications remain to be fully elucidated [15].

In Takayasu arteritis, the inflammatory process commences from the adventitia and progresses to the intima leading to segmental stenosis, occlusion, dilatation and aneurysm or aneurysm formation. Histologically, it is characterized as a 'panarteritis' involving all layers of the arterial wall, including intimal fibrous thickening and/or typical atheromatous lesions, destruction of medial smooth muscles and elastic layers, cellular infiltration and collagenous fibrosis in the media and thickened adventia with cellular infiltration around vasa vasorum. Intact areas between affected areas in arteries ('skipped lesions') are usually revealed in pathological studies [14].

Although the number of Takayasu arteritis patients is still limited, Takayasu arteritis is the most well studied PSV regarding the mechanisms of atheromatosis and arteriosclerosis. Most studies have demonstrated that carotid artery IMT (cIMT) is significantly higher in the Takayasu arteritis group as compared with the control group [16▪▪,19,20▪,21,22]. Moreover, high prevalence of atheromatic plaques is seen and can not be explained by the traditional vascular risk factors [16▪▪,18▪,19,20▪,21]. Of note, it was suggested that the abnormal cIMT might be used as a reliable marker of disease activity in Takayasu arteritis (sensitivity of 82% and specificity of 60%) and that it should be part of the routine evaluation of Takayasu arteritis [21,22]. In daily practice, clinicians may have difficulty in making differential diagnosis between Takayasu arteritis-related vascular lessions

Table 1. Noninvasive vascular biomarkers used for the study of atheromatosis and arteriosclerosis

	Intima–media thickness (IMT)	Plaque presence	Carotid–femoral pulse wave velocity (cfPMV)
Measured by	Ultrasonography	Ultrasonography	Applanation tonometry (most widely applied)
Disease studied	Arterial remodeling and/or atheromatosis (at the level of the common carotid artery)	Atheromatosis	Arteriosclerosis (arterial stiffening)
Arterial bed studied	Carotid	Carotid and femoral	Aorta (thoracic and abdominal)
Recommendation to be used in clinical practice in population at intermediate cardiovascular disease risk populations	Yes	Yes	Yes

and atheromatosis. Ugurlu *et al.* in a study of 58 patients investigated the morphologic and hemodynamic changes in the carotid arteries in Takayasu arteritis, along with patients with diabetes mellitus and healthy controls using Doppler ultrasonography (USG). The study showed that carotid artery may be helpful in differentiating Takayasu arteritis from atherosclerosis. Diffuse homogenous increase in IMT, presence of turbulence and higher resistivity index can be considered as suggestive of Takayasu arteritis rather than atherosclerosis [17▪▪].

Increasing data also suggest that Takayasu arteritis is associated with elevated arterial stiffness in the central elastic arteries compared with controls and that arterial stiffness may persist even when the disease is quiescent. PWV was found to be significantly increased in Takayasu arteritis patients, despite the younger age and the comparable blood pressure with the control group implying that structural rather than functional vascular damage takes place in Takayasu arteritis [15,23].

Giant cell arteritis

Giant cell arteritis (GCA), the most common granulomatous PSV with a predilection for large-sized and medium-sized arteries, occurs almost exclusively after the age of 50 years and affects mainly the extracranial branches of the carotid artery. It is associated with doubled incidence of cardiovascular events and 17 times increased risk for aortic aneurysms [24▪,25,26,31].

Similar to Takayasu arteritis, GCA inflammation involves all layers of the arterial wall and the inflammatory process appears to begin in the adventitial layer at the level of vasa vasorum. Granulomatous infiltrate including giant cells is proposed to play a key role in the invasion from the adventitial side to the medial and intimal layers [28].

Several early case reports (including few patients) describe a vasculitic carotid wall thickening in GCA [28], but there are also data showing significantly lower cIMT levels compared with controls [27]. A study of 41 GCA patients showed that steroid therapy has no influence on endothelial function but does significantly improves cIMT in GCA. Increasing data demonstrate that IMT measurement of temporal, facial and axillary arteries can correctly distinguish vasculitic from normal arteries in suspected GCA, thus IMT cut-off values may additionally help in the diagnosis of GCA [29▪]. Data for wall diameters are needed for future longitudinal trials to monitor GCA treatment.

Limited data are availiable concerning the potential effect of GCA on arterial stiffness. A study of 49 patients showed that GCA patients have higher PWV and dilated thoracic aortas with a women preponderance compared with GCA men. Further investigation is required to evaluate the effect of severity, treatment length, disease duration and cardiovascular risk factors on aortic morphology and function [30▪].

MEDIUM VESSEL VASCULITIDES

Polyarteritis nodosa

Polyarteritis nodosa (PAN) is an extremely rare, necrotizing vasculitis associated with aneurysmal nodules along the walls of medium-sized muscular arteries that can present initially as peripheral vascular ischemia [32,33]. Poor data, involving only a few patients have been found in the literature leading to inconclusive results for the development of accelerated atheromatosis [2,33,34,35,36].

Kawasaki disease

Kawasaki disease ia an acute medium vessel vasculitis, occuring predominantly in infants and during early childhood. The most significant complication is the development of coronary aneurysms during the subacute phase. These aneurysms are known to cause coronary artery disease by causing thrombosis and stenosis and represent a cause of sudden death in this patient group [37,38]. A review of autopsies from Kawasaki disease patients revealed that the arterial damage includes necrotizing arteritis, subacute or chronic vasculitis and luminal myofibroblastic proliferation [42▪].

Recent literature implicates in this, various factors like endothelial dysfunction, proatherogenic lipid profiles and arterial stiffening [38]. It is unclear whether all children with Kawasaki disease have increased later cardiovascular risk. The retinal microvasculature reflects changes in the microcirculation and is associated with traditional cardiovascular risk factors and events. Larger retinal venules may reflect chronic inflammation and endothelial dysfunction, and are associated with coronary artery disease in adults [39▪▪].

Carotid artery IMT is one of the most commonly used noninvasive measures of subclinical atherosclerosis in both pediatric and adult populations. There are few studies showing higher cIMT in children with Kawasaki disease compared with controls and others that demonstrate higher cIMT in patients of Kawasaki disease with coronary aneurysms [38]. As cIMT and aortic IMT have been shown to be a surrogate marker of both coronary and peritheral atherosclerosis, higher cIMT and aortic IMT in children with Kawasaki disease along with

proatherogenic abnormatiries in lipid profile may predict a higher risk of coronary artery events in later life [38,40▪▪,41▪▪,43▪].

Numerous studies have demonstrated the association between Kawasaki disease and arteriosclerosis. Overall patients with a history of Kawasaki disease exhibited a high PWV relative to controls. This suggests that these patients have a subsequent tendency for increased arterial stiffness. Consequently, life-long follow-up should be advised to evaluate cardiovascular diseases caused by former Kawasaki disease vasculitis and age-associated factors [37,44,45▪,46▪].

SMALL VESSEL VASCULITIDES

Antineutrophil cytoplasmic antibody-associated vasculitides

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) include three clinical entities: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPO), eosinophilic GPA (EGPA or Churg–Strauss syndrome). They are necrotizing vasculitis with few or no immune deposits affecting predominantly small vessels [1▪,4]. GPA and EGPA are also characterized by granulomatous inflammation [4]. Although AAV share several clinical and histopathological features, they represent three distinct diseases, mainly on the basis of ANCA antigen specificity [1▪].

Compared with the general population, the risk of cardiovascular disease (CVD; including MI, stroke and aneurysm formation) is two-fold to four-fold higher among patients with AAV, thus exhibiting enhanced cardiovascular mortality [3,7▪▪]. Patients with PR3 ANCA have a lower CVD risk than those with MPO ANCAs [47]. This is based on the fact that myeloperoxidase (a granule protein identified inside human atherosclerotic lesions and expressed in leucocytes) [49] has been implicated both in the initiation and progression of atheromatosis [7▪▪,48].

Several studies, most of which concern GPA [20▪,50,51▪] have shown increased common carotid IMT (ccIMT) in AAV patients as compared with controls [2,52], thus suggesting that AAV are associated with accelerated and frequently subclinical atheromatosis that can not be explained by traditional risk factors. Furthermore, the raised levels of high sensitivity C-reactive protein, MMPs and tissue inhibitor of metalloproteinase (TIMP) suggest that enhanced inflammation and excessive vascular remodelling are contributing factors in the development of accelerated atheromatosis in GPA [50]. González-Suárez *et al.* [53▪] in a study of 23 patients, observed an association between carotid intima–media thickness and internal carotid artery pulsatility index with small vessel cerebral disease pointing the possible use of carotid ultrasonography in predicting microvascular brain injury. Studies with a good number of MPO and EGPA patients were not found.

Only few data are availiable concerning the arterial stiffness as assessed by PWV measurement and the atherogenic index in AAV. Two studies with limited number of patients have shown increased arterial stiffness, and that arterial stiffness correlates with the degree of active inflammation in AAV patients [54,55].

As far as other small vessel vasculitides (i.e. purpura henoch schonlein and cryoglobulinemia vasculitis) are concerned, no reliable data showing accelerated atheromatosis and arteriosclerosis were found.

BEHCET'S DISEASE

Behcet's disease is a chronic, relapsing, multisystemic vasculitis involving both veins and arteries of any size [56]. In adition to the classic triad (recurrent apthous ulcers, genital ulcers, uveitis), pulmonary, gastrointestinal, nervous and musculoskeletal manifestations may be present [58▪▪]. Cardiovascular involvement (deep vein thrombosis, MI, arterial aneurysm, arterial thrombus formation) [59], occurs in 7–31% of patients and is associated with poor prognosis and increased mortality in patients with major vessel involvement [56].

Histopathologically, Behcet's disease is mainly characterized by vasculitis, with prominent neutrophil and monocyte infiltration in the perivascular regions with or without fibrin deposition in the vessel wall. Endothelial dysfunction, the initial lesion in atheromatosis, as well as the intermittent inflammation, autoimmune mechanisms and drugs are thought to account for the accelerated atherosclerosis in patients with Behcet's disease [58▪▪]. Of note, endothelial dysfunction in patients with Behcet's disease is modulated by the presence of corticosteroids and disease activity status. During disease relapse, corticosteroids restore endothelial dysfunction but their prolonged administration in the absence of active disease may be detrimental for the endothelium [57].

Despite previous evidence demonstrating atherosclerosis as not a prominent feature of Behcet's disease, even among patients with major organ involvement [60], a recent meta-analysis of relevant studies [61▪▪] showed that cIMT is increased in patients with Behcet's disease compared with controls. Similarly, the same meta-analysis showed that carotid plaques are three times more prevelent in

Table 2. Summary of data regarding the presence of accelerated atheromatosis and arteriosclerosis in primary systemic vasculitides

	Evidence of increased incidence of cardiovascular events	Evidence of accelerated atherosclerosis	Evidence of accelerated arteriosclerosis
Takayasu arteritis	++ [13,15]	++ [16▪▪,19,20▪,21,22]	+ [15,23]
Giant cell arteritis	++ [24▪,25,26,31]	++ [27,28,29▪]	+ [30▪]
Polyarteritis nodosa	+ [32,33]	□	□
Kawasaki disease	++ [37,38]	++ [38,40▪▪,41▪▪,42▪,43▪]	++ [37,44,45▪,46▪]
ANCA-associated vasculitides[a]	++ [3,7▪▪]	+ + [2,20▪,50,51▪,52,53▪]	+ [54,55]
Henoch Schonlein	□	□	□
Cryoglobulinemia vasculitis	□	□	□
Behcet's disease	++ [56,59]	+++ [61▪▪,62,63▪,64]	++ [58▪▪,65▪▪]

□, Lack of data; +, data derived from one or two single-center cohorts; ++, large number of evidence derived from multiple single-center cohorts; +++, evidence based on meta-analysis, ANCA, antineutrophil cytoplasmic antibodies.
[a]The evidence concerns granulomatosis with polyangiitis.

patients with Behcet's disease compared with the control group, verifying the presence of accelerated subclinical atheromatosis [58▪▪,61▪▪]. Another study including 50 patients showed that Behcet's disease may be associated with subtle increased cIMT, suggesting that it can be a predisposing factor for atherosclerotic arterial disease [62]. Further more, Cure *et al.* [63▪] in a cross-sectional case–control study, showed a strong positive correlation between atherogenic index of plasma (AIP) and cIMT. These and other results [64] suggest that accelerated subclinical atherosclerosis might explain the presence of increased cardiovascular events and mortality in these patients.

Moreover, several studies showed increased arterial stiffness, assessed by carotid to femoral PWV [58▪▪]. A study of 30 patients showed that PWV measurement might be more useful than cIMT in determination of vascular damage in Behcet's disease, especially in early stage of disease duration [65▪▪].

CONCLUSION

Despite the fact that there are no international networks dedicated to the study of cardiovascular risk factors, the role of PSV-drugs (e.g. corticosteroids) and vascular properties in PSV, recent studies using noninvasive techniques (cIMT and PWV) have demonstrated accelerated atheromatosis and increased arterial stiffening in these patients, thus suggesting a potential role for the increased cardiovascular events and associated mortality. Because of the limited number of PSV patients and the lack of prospective data and experience on vascular biomarkers by most rheumatologists, many PSV, especially PAN and small vessel vasculitides, remain under-studied (Table 2). The potential clinical value suggested by the so-far-studied vascular biomarkers would allow rheumatologists, implement optimal therapeutic strategies in the clinical practice in order to reduce the increased cardiovascular morbidity and mortality in PSV. This hypothesis prompts the need for large prospective cohorts that will record cardiovascular disease risk factors and apply the methods discussed herein, as well as other noninvasive methods, in order to provide useful future guidance regarding the evaluation and restratification of cardiovascular risk, which should lead to optimization of the prognosis and treatment of PSV patients.

Acknowledgements

None.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

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Antineutrophil cytoplasmic antibody-associated vasculitis and malignancy

Maria A.C. Wester Trejo, Ingeborg M. Bajema, and Emma E. van Daalen

Purpose of review
Patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) have an increased malignancy risk compared with the general population. This review aims to evaluate recent evidence for changes in the incidence of malignancy in patients with AAV and to examine explanations for the association between AAV and malignancy.

Recent findings
The overall malignancy risk in patients with AAV has decreased, most likely as a result of recent changes in therapeutic regimen, that is, a decrease in the exposure to cyclophosphamide. The risk of nonmelanoma skin cancer (NMSC), however, remains increased, which is probably attributable to treatment with azathioprine. Malignancy risk in patients with AAV treated with rituximab was found to be lower than in cyclophosphamide-treated patients. The incidence of malignancy prior to AAV is not increased compared with the general population.

Summary
Continuing efforts to reduce the exposure to cyclophosphamide have led to a decrease in malignancy risk in patients with AAV, except for NMSC. Rituximab could be a well tolerated alternative for cyclophosphamide regarding the development of malignancies.

Keywords
antineutrophil cytoplasmic antibody-associated vasculitis, immunosuppressive therapy, malignancy

INTRODUCTION

Over the past two decades, a significant number of studies have tried to understand the link between antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) and malignancy development. In 1992, Hoffman *et al.* [1] were the first to find an increased malignancy risk in patients with granulomatosis with polyangiitis (GPA). Subsequent studies found increased risks of, in particular, bladder cancer, leukemia and nonmelanoma skin cancer (NMSC) [2–8]. The increased malignancy risks have been largely attributed to the well known carcinogenic effects of cyclophosphamide [9]. Also, azathioprine and TNF-α inhibitors have been associated with an increased risk of malignancies in patients with AAV, in particular with respect to NMSC and solid malignancies [6,7]. In addition to the malignant side effects of immunosuppressive therapy, it has been suggested that chronic stimulation of the immune system because of AAV may contribute to the increased malignancy risk [10]. Alternatively, some studies found an increased malignancy risk prior to the diagnosis of AAV, suggestive of common pathogenic pathways in both diseases [11,12]. The latter hypothesis would implicate that patients with AAV have an intrinsically higher malignancy risk compared with the general population, irrespective of immunosuppressive therapy.

As therapeutic regimens for AAV have changed tremendously over the past 10 years, more recent studies were warranted to investigate possible changes in malignancy risks in patients with AAV. Alternative explanations for the development of malignancies in addition to therapeutic side effects have not been fully elucidated. Therefore, this review aims to discuss recent data, published in the last 2 years, on the incidence of malignancy in patients with AAV, its relationship with therapies,

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Curr Opin Rheumatol 2018, 30:44–49
DOI:10.1097/BOR.0000000000000448

KEY POINTS

- Continuing efforts to reduce exposure to cyclophosphamide over the years have led to a decrease in the risk of developing malignancies in patients with AAV.
- Currently, the increased malignancy risk in patients with AAV is solely driven by an increased risk of developing NMSC, possibly associated with azathioprine use. This warrants regular skin cancer screening and skin protection from ultraviolet radiation.
- First data indicates that the use of rituximab is a well tolerated alternative to cyclophosphamide in the treatment of AAV with regard to the development of malignancies.
- The malignancy risk prior to the diagnosis of AAV is not increased compared with the general population, not supporting the hypothesis that malignancy and AAV have shared pathogenic pathways.

as well as the putative association between malignancies and ensuing AAV.

CHANGES IN MALIGNANCY INCIDENCE OVER TIME: A ROLE FOR CYCLOPHOSPHAMIDE

In 2013, Mahr *et al.* [13] comprehensively reviewed earlier studies (1992–2011) investigating malignancy incidence in patients with AAV. Most studies found a significantly increased overall malignancy risk in patients with AAV compared with the general population, with standardized incidence ratios (SIR, i.e. a ratio indicating the risk of patients compared with that of the sex-matched, age-matched, and calendar-year matched general population) ranging from 1.6 to 2.0. The malignancy types most consistently linked to AAV were bladder cancer (SIR 2.4–33), leukemia (SIR 3.2–5.9) and NMSC (SIR 2.8–10.4). Recently, Shang *et al.* [14▪] have been the first to perform a meta-analysis investigating the relationship between malignancy and AAV. This meta-analysis found a significantly increased overall malignancy risk in patients with AAV compared with the general population with a SIR of 1.74 (95% CI 1.37–2.21). It was confirmed that the malignancies most frequently observed in AAV were NMSC (SIR 5.18, 95% CI 2.72–5.42), leukemia (SIR 4.89, 95% CI 2.93–8.16) and bladder cancer (SIR 3.84, 95% CI 2.72–5.42). These were followed by lymphoma (SIR 3.79, 95% CI 1.87–7.69), liver cancer (SIR 3.50, 95% CI 1.45–8.43) and lung cancer (SIR 1.67, 95% CI 1.07–2.60). Previously reported increased risks of kidney, prostate, colon and breast cancer were not validated in this meta-analysis.

The increased malignancy risk in patients with AAV has been largely attributed to the well-established carcinogenic effects of cyclophosphamide. Its use as an immunosuppressive agent has been related to bladder cancer and leukemia in a range of contexts including not only AAV, but also, for example, rheumatoid arthritis and lymphoma [9]. The causal relationship between cyclophosphamide exposure and the development of malignancy in AAV became more firmly established when two studies found a dose–response relationship [2,15]. Several years ago, Faurschou *et al.* [2] examined the incidence of various malignancies among 293 patients with GPA stratified according to cyclophosphamide exposure in Denmark. Patients were diagnosed between 1973 and 1999 and the median duration of follow-up was 6 years. An increased incidence of NMSC, bladder cancer and myeloid leukemia was found among those patients treated with high cumulative cyclophosphamide doses (>36 g). Recently, Faurschou *et al.* [16▪] revisited this cohort after extended follow-up throughout 2010, with a median follow-up of 9.7 years. Especially because the survival time of patients with AAV has improved, long-term effects of treatment have become increasingly relevant [17]. With this in mind, their aim was to characterize the long-term malignancy risk in patients with GPA and its relation to the conventional therapies received at the time of diagnosis. Notably, a novel finding was the significantly increased risk of very late occurring NMSC and bladder cancer. The risk of NMSC was increased from the second year of follow-up onwards, with a SIR of 7.0 (95% CI 2.3–16) after 20 years. The SIR for bladder cancer was 5.3 (95% CI 5.3–31.0) after 5–9 years of follow-up, increasing to 14.4 (95% CI 5.3–31) after 10–14 years and 10.5 (95% CI 1.2–38.0) after 15–19 years.

As a result of treatment-related concerns, and in order to improve therapeutic efficacy, international randomized controlled trials have been conducted since the 1990s, which have led to changes in the standard therapy of AAV [18–21]. One of the most important changes was based on the results from the CYCAZAREM trial published in 2003, concluding that switching from cyclophosphamide to azathioprine at the time of remission did not lead to an increase in disease relapse and was, therefore, a well tolerated alternative to prolonged cyclophosphamide exposure [20]. Rahmattulla *et al.* [22▪] examined the incidence of malignancy in 138 patients with AAV diagnosed between 1991 and 2013 in the Netherlands, thereby investigating patients who were treated with more recent therapy regimens, in contrast to the cohort from Faurschou *et al.* [16▪]. Interestingly, they found a significantly increased malignancy risk (SIR 2.43, 95% CI 1.76–3.26) in

patients diagnosed before 2003, that is, before the publication of CYCAZAREM, whereas patients diagnosed in or after 2003 did not have an increased malignancy risk (SIR 1.34, 95% CI 0.49–2.92). The duration of cyclophosphamide therapy was significantly lower in patients diagnosed in or after 2003, supporting the earlier described dose–response relationship between cyclophosphamide and malignancy risk [15,16▪]. Additionally, a subanalysis showed that patients positive for ANCA directed against proteinase 3 (PR3-ANCA) had a significantly increased malignancy risk (SIR 2.72, 95% CI 1.80–3.93), in contrast to patients positive for ANCA directed against myeloperoxidase (MPO-ANCA) (SIR 1.87, 95% CI 0.93–3.34). Survival in both groups was similar; however, the duration of cyclophosphamide treatment was significantly longer in patients with PR3-ANCA positivity, again pointing towards a dose–response relationship and, consequently, a possible causal association.

Another important finding in the study by Rahmattulla *et al.* [22▪] was that the overall increased malignancy risk was mainly driven by an increased risk of NMSC (SIR 4.23, 95% CI 2.76–6.19). This finding was similar to the one reported in the 5-year follow-up study of patients initially recruited in four European Vasculitis Society (EUVAS) clinical trials; the risk of NMSC was significantly increased (SIR 2.78, 95% CI 1.56–4.59), but increased risks of bladder cancer and hematological malignancies were not observed [3]. The remaining increased risk of NMSC has been linked to the use of azathioprine, not only in patients with AAV [7,22▪], but also in patients with other autoimmune disease entities and after transplantation [9,23–27]. A possible explanation is that azathioprine sensitizes the skin to ultraviolet A radiation [28]. Additionally, increased risk of skin cancer has also been linked to the use of corticosteroids [29]. Therefore, the increased risk of NMSC in AAV could be a result of the use of not only cyclophosphamide, but of a combination of immunosuppressive therapies. Unfortunately, data on the cumulative dose of other immunosuppressive agents, such as corticosteroids and azathioprine, are difficult to retrieve, making it impossible to assess their influence on malignancy risk. The increased risk of NMSC warrants regular screening for skin cancer and protective measures against exposure to ultraviolet radiation in patients receiving immunosuppressive therapy.

RITUXIMAB AS ALTERNATIVE IMMUNOSUPPRESSIVE AGENT: EFFECT ON MALIGNANCY

In the search for less cytotoxic and more effective AAV treatment regimens, rituximab has emerged as a promising alternative for cyclophosphamide and has recently been included in the European League against Rheumatism (EULAR) recommendations for AAV management [30–32]. The initial findings from randomized controlled trials investigating the use of rituximab in AAV indicated that treatment efficacy was comparable between rituximab and cyclophosphamide [21,33,34]. On the basis of the first data, however, some concerns were raised about a possible higher rate of malignancies in patients treated with rituximab [35,36].

A recent study by van Daalen *et al.* [37▪▪] investigated the long-term malignancy risk in 323 patients with AAV diagnosed between 2000 and 2014 in the United Kingdom, comparing rituximab-treated patients with cyclophosphamide-treated patients. In accordance with the two recent studies described above [16▪,22▪], overall malignancy risk was significantly increased during a mean follow-up of 5.6 years (SIR 1.98, 95% CI 1.38–2.53); however, the risk of malignancies excluding NMSC was comparable with that of the general population (SIR 1.09, 95% CI 0.67–1.69). The study confirmed the increased malignancy risk in patients treated with cyclophosphamide. In contrast, rituximab-treated patients had a malignancy risk similar to that of the general population (SIR 0.67, 95% CI 0.08–2.43). When comparing the two treatment groups, patients treated with cyclophosphamide had a 4.61-fold higher risk (95% CI 1.16–39.98) of malignancy than those treated with rituximab. Patients treated with both cyclophosphamide and rituximab had a lower malignancy risk than patients treated with only cyclophosphamide (SIR 1.01, 95% CI 0.46–1.93), although the mean cumulative cyclophosphamide dose was similar between the two groups. Moreover, a nonsignificant trend was seen towards an inverse dose–response relationship between the cumulative rituximab dose and malignancy risk. The higher the cumulative rituximab dose, the lower the malignancy risk, with a 6.32-fold lower malignancy risk (95% CI 1.99–32.15) in patients treated with more than 6.0 g rituximab compared with patients who did not receive rituximab. This remarkable finding could point towards a possible protective role of rituximab in the development of malignancies. However, more and larger studies are required to validate this finding. More conclusive data on this topic may result from the Rituximab in ANCA-associated Vasculitis Registry (RaVeR) study: an ongoing open-label real-world study of patients with GPA and microscopic polyangiitis (MPA) treated with rituximab. In a recent interim analysis of this study, based on a total of 97 patients with a median follow-up of 2.4 years, crude incidence rates of adverse events including

Table 1. Malignancy risk in antineutrophil cytoplasmic antibody-associated vasculitis

Study	Hoffman *et al.* 1992 [1]	Knight *et al.* 2002 [5]	Westman *et al.* 1998 [7]	Faurschou *et al.* 2008 [2]	Holle *et al.* 2011 [4]	Heijl *et al.* 2011 [3]	Zycinska *et al.* 2013 [8]	Shang *et al.* 2015 [14▪]	Faurschou *et al.* 2015 [16▪]	Rahmattulla *et al.* 2015 [22▪▪]	van Daalen *et al.* 2017 [37▪▪]
SIR overall (95% CI)	2.4 (not reported)	2.0 (1.7–2.5)	1.6 (0.9–2.7)	2.1 (1.5–2.7)	0.8 (0.5–1.4)	1.6 (1.2–2.1)	2.5 (1.2–2.9)	1.74 (1.37–2.21)	1.9 (1.5–2.4)	Overall: 2.21 (1.64–2.92) Before 2003: 2.43 (1.76–3.26) In or after 2003: 1.34 (0.49–2.92)	CYC: 3.10 (2.06–4.48) RTX: 0.67 (0.08–2.43)
SIR NMSC (95% CI)	Not reported	7.3 (4.4–12)	10.4 (3.4–24.3)	4.7 (2.8–7.3)	Not reported	2.8 (1.6–4.6)	5.2 (2.3–8.7)	5.18 (3.47–7.73)	4.0 (2.7–5.7)	4.23 (2.76–6.19)	4.58 (2.96–6.76)
SIR Leukemia (95% CI)	Not reported	5.7 (2.3–12)	Not reported	5.9 (1.2–17)	Not reported	3.2 (0.4–11.7)	4.3 (2.1–11.2)	4.89 (2.93–8.16)	13.3 (3.6–34)	Not observed	Not observed
SIR bladder cancer (95% CI)	33 (not reported)	4.8 (2.6–8.1)	4.8 (1.0–13.9)	3.6 (1.2–8.3)	Not reported	2.4 (0.7–6.2)	3.4 (1.6–5.2)	3.84 (2.72–5.42)	5.5 (2.7–9.8)	1.43 (0.04–7.96)	1.53 (0.04–8.57)
Study period	~1967–1990	1969–1995	1971–1996	1973–1999	1966–2005	1995–2007	1990–2008	1967–2008	1973–1999 follow-up 2010	1991–2013	2000–2014
Median follow-up (years)	8	Not reported	4.58	6	Not reported	4.95	7	4.58–7	9.7	10	5.6
Number of patients	158	1065	123	293	445	535	117	2578	293	138	323
Number of malignancies	Not reported	110	15	50	18	50	15	258	73	85	45
Study design	Monocenter clinical cohort	Nationwide hospital discharge database study	Monocenter clinical cohort study	Nationwide hospital discharge database study	Monocenter clinical cohort study	Long-term follow-up of multicenter clinical trials	Monocenter clinical cohort study	Meta-analysis including all studies before 2015 except Hoffman *et al.*	Long-term follow-up of nationwide hospital discharge database study	Monocenter clinical cohort study	Monocenter clinical cohort study

CYC, cyclophosphamide; RTX, rituximab; SIR, standardized incidence ratio.

malignancy were not increased compared with similar patient cohorts with renal involvement [38].

Of note, the study by van Daalen *et al.* [37▪▪] was the first to include patients with eosinophilic granulomatosis with polyangiitis (EGPA) in addition to GPA and MPA, the former having a lower incidence but treated similarly [32]. The EGPA subgroup had a 2.75-fold increased malignancy risk (95% CI 1.19–5.40) compared with the general population. Therefore, patients with EGPA should be monitored and counseled with regard to malignancy in the same way as patients with MPA and GPA.

ALTERNATIVE EXPLANATIONS FOR INCREASED MALIGNANCY RISK IN ANTINEUTROPHIL CYTOPLASMIC ANTIBODY-ASSOCIATED VASCULITIS

Recent findings strongly suggest that the observed decrease in malignancy risk in AAV over time is a result of continuing efforts of reducing the exposure to cyclophosphamide (see Table 1 for an overview of studies). A suggested alternative explanation for the increased risk of malignancy in patients with AAV, other than through immunosuppressive therapy, is prolonged chronic stimulation of the immune system because of vasculitis itself [10]. As longer and more severe disease activity is accompanied by longer and more intensive immunosuppressive treatment, it would be impossible to tease these effects apart.

In addition to therapeutic side effects and chronic immune stimulation, another explanation for the link between malignancy risk and AAV has been suggested, namely that these conditions have common pathogenic pathways [12,39]. In this scenario, patients with AAV would have an intrinsically higher risk to develop malignancies, based on a state of acquired immunological dysfunction underlying both conditions [39,40]. This has been studied by examining malignancies preceding the onset of AAV, that is, before therapy with immunosuppressive agents has started. Two studies found an increased malignancy risk preceding the diagnosis of AAV [11,12], whereas another study contradicted these results [39]. In a recent study on hematologic malignancy (mainly non-Hodgkin's lymphoma and myelodysplasia) it was reported that 16 out of 2244 (0.7%) patients with AAV had a hematologic malignancy and in 5 of these patients the malignancy preceded the onset of AAV [41]. The authors did not compare this with the incidence of hematologic malignancy in the general population, but concluded that the association was unlikely to be fortuitous despite its rarity. They emphasize the need to take into account both diseases to ensure effective treatment in patients with both diagnoses.

In order to analyze malignancy risk prior to AAV diagnosis, a recent study was performed in 203 patients with GPA or MPA in the Netherlands [42▪]. They investigated the incidence of malignancy preceding AAV in comparison with the incidence in the general population matched for sex, age and time period, thereby being the first to take into account SIRs in this context. The overall malignancy risk preceding AAV diagnosis was comparable with that of the general population (SIR 0.96, 95% CI 0.55–1.57), as was the case for different malignancy types analyzed separately. Additionally, no difference in malignancy risk was found between GPA and MPA patients. These results do not support the hypothesis that AAV and malignancy have shared pathogenic pathways. Therefore, routine screening for underlying malignancies in patients newly diagnosed with AAV seems unnecessary.

CONCLUSION

After the introduction of cyclophosphamide as a treatment for AAV, patients showed dramatically increased survival at the cost of an increased risk of bladder cancer, leukemia and NMSC directly related to cyclophosphamide dose. With ongoing efforts to diminish the exposure to cyclophosphamide, these malignancy risks in patients with AAV have decreased. Currently, patients with AAV receiving immunosuppressive therapy only show an increased risk of developing NMSC, warranting skin cancer screening and protection of the skin against ultraviolet radiation. Rituximab seems a well tolerated alternative to cyclophosphamide with regard to the development of malignancies, and first data points towards a possible protective effect against malignancy. The malignancy risk preceding the diagnosis of AAV, that is, before therapy with immunosuppressive agents has started, is not increased compared with the general population.

Acknowledgements

The authors thank Jan Schoones for his help with a database literature search.

Financial support and sponsorship

E.E.v.D is a PhD student supported by the Kolff Student Researcher Grant from the Dutch Kidney Foundation.

Conflicts of interest

There are no conflicts of interest.

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This study investigated malignancy risk preceding onset of AAV, compared with the general population. No significant difference was found, not supporting the hypothesis that AAV and malignancy share common pathogenic pathways and making routine screening for underlying malignancies in patients with newly diagnosed AAV seem unnecessary.

A review of current management of vasculo-Behcet's

Mira Merashli, Rozana El Eid*, and Imad Uthman*

Purpose of review
To give an overview of recently published articles about the management of vasculo-Behcet's with particular emphasis on anticoagulation.

Recent findings
Biologic agents are emerging as a potential therapeutic option in refractory vasculo-Behcet with a good safety profile. Evidence further shows that following nonpulmonary aneurysm repair, there is a reduced risk of recurrent aneurysmal formation at the operative site in patients treated with immunosuppressants in addition to their surgery, than those undergoing surgical intervention alone.

Summary
Behcet disease patients are at risk of developing multiple vascular complications including thrombosis and aneurysms. Treatment should focus on reducing inflammation; and the role of anticoagulation is still debatable.

Keywords
anticoagulation, Behcet's disease, thromboembolism, treatment, vascular disease

INTRODUCTION

Behçet's disease is a systemic autoinflammatory disorder characterized by recurrent oral ulcers, genital ulcers, ophthalmologic, musculoskeletal, vascular and cutaneous manifestations [1]. It is a rare disease in North America and Europe, and is primarily found along the ancient Silk Road that extends from the Mediterranean region to Eastern Asia. A recent meta-analysis showed prevalence proportions (expressed as cases/100 000 inhabitants) as 10.3 for all studies and 119.8 for Turkey, 31.8 for the Middle East, 4.5 for Asia, and 3.3 for Europe [2]. Behçet's disease is highly heterogeneous, manifesting clinically with different ways depending on the geographic distribution. Vascular involvement is usually either in the form of thrombosis or aneurysm. The aforementioned vasculopathies present a dilemma of whether or not anticoagulation is warranted. The aim of this study is to describe the treatment possibilities for Behçet's disease in the setting of vascular thrombosis and/or aneurysms, with focus on the role of anticoagulation.

SEARCH STRATEGY AND SELECTION CRITERIA FOR REVIEW

We searched Medline and PubMed matching the key search terms vascular disease, thromboembolism, anticoagulation, and Behcet's Disease. Full texts, as well as abstracts of published articles were reviewed, focusing on those published from February 2016 to 2017.

CLINICAL PICTURE AND PATHOGENESIS OF VASCULAR-BEHCET

Vascular involvement is present in up to 40% of cases, 75% of which are venous in nature, whereas the other 25% are arterial [3].

Lower extremity venous thrombosis is the most common [3], though vena cava thrombosis, pulmonary artery aneurysms, Budd–Chiari syndrome, peripheral artery aneurysms, dural sinus thrombosis, and abdominal aortic aneurysms (AAAs) do also occur [4].

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Curr Opin Rheumatol 2018, 30:50–56
DOI:10.1097/BOR.0000000000000458

KEY POINTS

- Glucocorticoids, azathioprine, cyclophosphamide, or cyclosporine A are the recommended first line treatments in vasculo-Behcet.
- Infliximab, alemtuzumab, and adalimumab are the only biologic agents documented to have efficacy in treatment of vascular manifestations.
- Vascular involvement is present in up to 40% of cases, 75% of which are venous in nature, whereas the other 25% are arterial.

Cardiovascular pathology in Behçet's disease is thought to be caused by both immunological and inflammatory factors that induce a hypercoagulable state and endothelial damage. The vasculitis which is a hallmark of Behçet's disease, can further augment platelet aggregation, and impair fibrinolysis resulting in thrombosis [5].

In contrast to other prothrombotic diseases, systemic inflammation in Behçet's disease plays a major role in pathogenesis, whereas other thrombophilic factors play a secondary role [6].

Though the pathogenesis of thrombus formation is unclear, inflammation-mediated thrombosis may be explained partly by immune dysfunction in addition to the genetic polymorphisms in hemostatic factors (Fig. 1).

A hypersensitivity of T lymphocytes to different types of antigens, as well as overproduction of proinflammatory cytokines by T cells and neutrophils, have been reported [7]. In addition, interleukin 21 and interleukin 17A-producing T cells seem to be involved in the inflammatory process by promoting T-helper (Th)1 and Th17 differentiation and regulatory T cells suppression, which correlates with Behçet's disease activity [8]. However, interleukin 21 blockade with an interleukin 21 recombinant human interleukin 21 receptor/Fc Chimera Protein restored the TH17 and regulatory T-cells' homeostasis in patients with Behçet's disease [8].

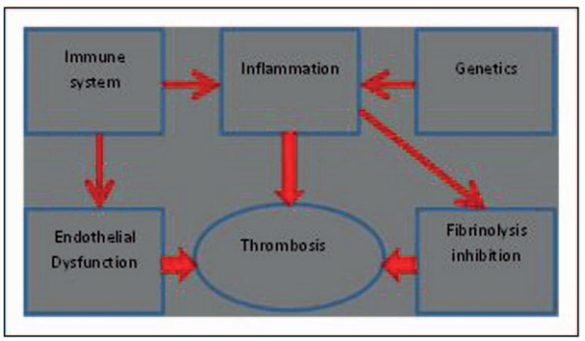

FIGURE 1. Thrombosis is a result of multifactorial derangements in systemic inflammation, fibrinolysis, and the endothelium. The immune system and genetics indirectly affect thrombosis through augmenting inflammation and endothelial dysfunction.

Genetic polymorphisms in coagulation factors have also been studied in Behçet's disease, suggesting that there may be an additional risk factor for thrombosis in certain populations. Controversial data has been reported about the role of some procoagulant factors, such as prothrombin G20210A polymorphisms [6],with many studies stating the prothrombin gene mutation was not pertinent [9].

Furthermore, genetic susceptibility has been clearly demonstrated in patients with systemic vasculitis, including Behçet's disease. A recent study examining 19-independent genetic susceptibility variants identified using genome-wide association studies has found epigenomic effects on hematopoietic stem cells in Behçet's disease as well as involvement of multiple cell types, as opposed to specific cell subsets like in Kawasaki disease [10]. A surprising finding of a role for B cells is in the pathogenesis of Behçet's disease warrants further investigation for targeted therapies [10].

Impaired fibrinolysis in Behçet's disease could be explained by the elevated levels of lipoprotein(a) found in Behçet's disease patients [11], as well as high plasma levels of thrombin-activable fibrinolysis inhibitor reported in some studies [12].

Inflammation and immune derangement may further cause damage to endothelial cells, contributing to the prothrombotic state. Evidence of endothelial cell dysfunction, comes from studies reporting a declined production of nitric oxide, a prominent marker of endothelial dysfunction [13]. Other endothelial injury markers, such as circulating von Willebrand factor [14] and thrombomodulin [15] were found to be elevated in patients with active Behçet's disease. Increased serum levels of some adhesion molecules such P selectin, was also described. Located in the Weibel–Palade bodies of endothelial cells and in the granules of platelets and released into the plasma during platelet activation, P selectin promotes inflammatory reactions by enabling leukocyte recruitment at the injury site [16]. The immune system in addition to promoting inflammation, may contribute to endothelial cell dysfunction, by producing antiendothelial cell antibodies [17].

As a result, the peculiar feature of prothrombotic state in Behçet's disease is that chronic relapsing thrombotic events transform veins into fibrotic structures with a constricted lumen and sticky thickened vessel walls, forming a thrombus strongly adherent to the vessel wall with a low possibility of embolism [18].

In addition, and quite importantly, evidence points to the implication of neutrophils in the development of thrombosis. A study collected blood samples from 98 Behçet's disease patients, and found altered fibrinogen structure and impaired function that significantly correlated with neutrophil activation and subsequent increased reactive oxygen species (ROS) production [19▪▪].

On the other hand, arterial lesions in Behçet's disease can be occlusive or aneurysmal. They are commonly isolated but may be multiple [20]and frequently coexist with venous thromboses [21]. The most feared complication is aneurysm formation and rupture [22]. The abdominal aorta is one of the most common sites of aneurysm formation, and approximately 60% of reported arterial lesions associated with Behçet's disease are aneurysms [23].

The pathogenesis of aneurysm formation in Behçet's disease is different than for atherosclerotic aneurysms. In Behçet's disease, vasculitis leads to destruction of the media, arterial dilatation, and fibrosis with subsequent inflammatory destruction of the vasa vasorum [22]. This results in transmural necrosis and consequently pseudoanurysm formation or rupture in the arterial wall [22]. A characteristic feature of aneurysm in Behçet's disease is its tendency for recurrence, which can occur in different vessels [22].

Increased serum levels of matrix metalloproteinase proteins (MMP), MMP-9 and MMP-2, were highly associated with vasculo-Behçet's disease, particularly aneurysmatic involvement [24]. Thus, MMPs, which play an important role in tissue remodeling and destruction of extracellular matrix components, may have an important role in aneurysm formation.

TREATMENT

General considerations

Though the specific etiopathogenesis of Behcet's disease is not established well, evidence supports the inflammatory nature of the syndrome. Management options are largely confined to symptomatic treatment, mostly involving manipulation of the immune system, and modified based on the severity of the illness and the constellation of symptoms that accompany it.

The main aim is to avoid fatal events such as rupture of aneurysm and pulmonary thromboembolism, and to relieve various symptoms related to vascular involvement.

The European League against Rheumatism (EULAR) suggests the use of glucocorticoids, azathioprine, cyclophosphamide, or cyclosporine A because of their effectiveness in promoting a quick and persistent suppression of the vascular lesions and to avert the expansion of thrombosis and its recurrence [25]. Recommendations are summarized in Table 1. Of note, there is currently no US Food and Drug Administration approved treatment for Behcet's disease.

Anticoagulation and antiplatelet agents

A well established rule in modern medicine is to treat venous thrombosis with anticoagulation. The International Collaboration of Aspirin Trials for Recurrent Venous Thromboembolism (INSPIRE) collaboration found that aspirin after anticoagulant treatment reduces the overall risk of recurrence by more than a third in a broad cross-section of patients with a first unprovoked venous thromboembolism, without significantly increasing the risk of bleeding [26]. Furthermore, experimental data has shown that antiplatelet therapy may decrease thrombus formation, reduce aortic wall inflammation and stabilize the aortic wall in AAAs [27]. Though the volume of thrombus within AAAs does parallel the maximum AAA diameter [28] still, a definitive link between platelet inhibition and aneurysm progression has not been identified [29]. On the other hand, there is currently no evidence to suggest that aspirin therapy contributes to initiating AAA expansion or rupture.

Table 1. Treatment of major vessel disease in Behçet's disease

Drug	Recommendation
Corticosteroids	Management of acute deep vein thrombosis in BD. Management of pulmonary and peripheral arterial aneurysms
Azathioprine	Management of acute deep vein thrombosis in BD
Cyclophosphamide	Management of acute deep vein thrombosis in BD. Management of pulmonary and peripheral arterial aneurysms.
Cyclosporine A	Management of acute deep vein thrombosis in BD
Antiplatelet/ anticoagulation	No controlled data or evidence of benefit of antiplatelet, anticoagulant, or antifibrinolytic agents in management of deep vein thrombosis

BD, Behçet's disease.
Adapted with permission [24].

However, because of the aforementioned differences in the pathophysiology of the vasculo-Behcet, namely the low risk of embolization, most experts recommend against using anticoagulation or aspirin [1,25].

To date, there have been no randomized control studies documenting the risk versus benefit of anticoagulant or antiplatelet therapy. Further complicating this issue is that in Behçet's disease there may be concurrent occult vascular pathologies such as hidden aneurysms or vascular defects, whereby anticoagulation would be contraindicated.

Observational studies involving retrospective cohorts and case studies show mixed results.

In a study of patients with Behçet's disease from 15 rheumatology centers in Turkey, who were given immunosuppressants and anticoagulation treatment, only minor hemorrhage related to anticoagulation was observed in 4.7% patients [30]. Furthermore, multivariate analysis showed that development of vascular relapse negatively correlated with immunosuppressant treatments alone; however, there was no additional positive effect when used with anticoagulation [30]. In addition, other retrospective studies have supported the conclusion that recurrence of venous thrombosis is significantly reduced in patients receiving immunosuppressive agents, whereas anticoagulants were not associated with reduced risk [31,32].

However, in another investigation, it was found in a cohort of 52 patients with cardiovascular involvement that complete remission of cardiac involvement was associated with the use of anticoagulants (47.6 versus 12.9%) [33].

Further complicating the issue is that there are also no guidelines for the use of anticoagulants in the perioperative period of aneurysm repair. Following 51 surgical interventions, in one study, all patients were placed on long-term, low-dose aspirin and an anticoagulant was initiated to avoid thromboembolic complications after stent placement in patients with peripheral arterial aneurysm, however adverse bleeding events were not reported [34▪].

Traditional agents

Glucocorticoids

The mainstay of management in any autoinflammatory disease is glucocorticoid therapy, and this principle has been extended to the treatment of medium–high severity Behçet's disease. Glucocorticoids have a wide array of mechanisms by which they inhibit inflammation promoting its resolution. Most notably through inhibiting secretion of inflammatory cytokines such as interleukin 1, interleukin 2, interleukin 6, interleukin 8, tumor necrosis factor (TNF), and granulocyte-macrophage-colony-stimulating factor and also interfering with leukocyte migration. Some immunologic effects of glucocorticoids are dose dependent. In the low to moderate dose range, T lymphocytes may be somewhat in the circulation, and delayed type hypersensitivity responses may be impaired. At higher doses, lymphocyte proliferation to mitogens was maximally suppressed *in vitro*.

There are no randomized controlled trials (RCTs) demonstrating the efficacy of corticosteroids in the treatment of vasculo-Behcet. Nevertheless, the EULAR recommends early initiation of corticosteroids for the management of severe or life-threatening ocular, vascular, gastrointestinal, or neurologic disease [25]. In that case, pulse-dose steroids (1 g intravenous methylprednisolone infusions daily) are often used for 3 days, followed by 1 mg/kg/day prednisolone tapered slowly [35].

Azathioprine

Azathioprine is one of the most studied immunosuppressive drugs that subdues both cellular and humoral immune responses. Azathioprine's active metabolite methyl-thioinosine monophosphate is a purine synthesis inhibitor that works by blocking the enzyme amidophosphoribosyl transferase and, thus, impairs DNA synthesis which especially targets fast-growing cells without a method of nucleotide salvage such as lymphocytes. Though, the only RCT conducted with azathioprine establishes its benefit with ocular involvement in Behçet's disease, the efficacy with vascular lesions is less clear [36]. Despite this, azathioprine is recommended for the treatment of pulmonary aneurysms after treatment with cyclophosphamide for at least 2 years [25]. Azathioprine (2.5 mg/kg/day) may be prescribed for venous thrombosis of the extremities [25].

Cyclophosphamide

The EULAR recommends this agent in both central or peripheral arterial aneurysm and venous thrombosis. As a more potent immunosuppressant monthly pulses of cyclophosphamide, may be preferred for thrombosis of the superior vena cava or Budd–Chiari syndrome.

Though few studies have been conducted, evidence shows cyclophosphamide has a good outcome in neurological and vascular manifestations, but not in ocular pathology [37]. It exerts its mechanism of action mainly through its metabolite phosphoramide mustard, which is only formed in cells that have low levels of aldehyde dehydrogenase gene. Phosphoramide mustard forms irreversible DNA crosslinks both between and within DNA strands which leads to cell apoptosis.

Cyclosporine A

This agent acts through inhibition of interleukin 2 and interleukin 17, and is usually used in combination with glucocorticoids [38]. Several investigations have been conducted with cyclosporine A, including three RCTs comparing its efficacy against cyclophosphamide [39], colchicine [40], and conventional therapy (steroid or chlorambucil) [41]. All three studies supported its efficacy in treating ocular manifestations of Behçet's disease. However, there is also a documented risk of neurotoxicity associated with its use [42]. Though its benefit for vascular disease is less studied, there has been one open-label study in which seven patients with venous thrombosis had complete resolution of thrombophlebitis without residual venous insufficiency and no recurrences as long as treatment was continued [43]. As such the EULAR has recommended that cyclosporine A may be used in the management of acute deep vein thrombosis, except in the case of central nervous system involvement unless necessary for intraocular inflammation [25].

Surgical treatment

Surgery of venous thrombosis leads to many complications; therefore, it is reserved only for the treatment of arterial involvement, namely aneurysms [44]. In contrast to atheromatous aneurysms the indications and ideal timing of surgery for Behçet's et's disease-related aneurysms is controversial. On one hand, some believe that aneurysms attributable to Behçet's disease have a higher possibility of rupture with no connection between the size of the aneurysm and risk of rupture and as such suggest aggressive and invasive management as soon as the diagnosis is established [45]. However, there is also a marked incidence of complications postoperatively; hence, other authors recommend against surgical intervention in patients with small, intact, saccular aneurysms [46,47]. It is important to note that several studies [34▪,48] have shown that postoperative complications were reduced in patients receiving corticosteroids and immunosuppressants.

An endovascular approach is minimally invasive and effective for aortic aneurysms [34▪,49]; however, one study found that bypass surgery may deliver improved outcomes for extremity arterial aneurysms than placement of endovascular stents [34▪]. Endovascular embolization can be beneficial for the treatment of pulmonary artery involvement, in medical treatment refractory cases [44]. Chronic thromboembolic pulmonary hypertension may also be a potential complication, though Behçet's disease is not considered a risk factor for this condition in general [50▪]. In this situation, the only treatment is pulmonary endarterectomy, and so far only one case report and one cohort study of nine patients has reported on the outcomes of pulmonary endarterectomy. In the latter study, no perioperative mortality was observed; however, two patients had significant morbidity [50▪]. Thus, the authors recommended this option for thrombotic lesions that are surgically accessible and undertaken in centers with experience [50▪].

Biologic agents and monoclonal therapy

Biologic agents are those that are made from a living organism or its products, such as anti-TNF α (infliximab, etanercept, and adalimumab), B-cell depleting agents (rituximab), and specific mAbs to interleukin 1 (anakinra) and interleukin 6. Though much research has been conducted recently with regards to their use in treating various rheumatologic disorders, this review will focus on the drugs shown to be effective in vasculo-Behcet. Mostly, biologic agents are used in Behçet's disease refractory to conventional immunosuppression [51].

For infliximab, published data addressing vascular involvement is limited to case reports; however, the results point toward the efficacy of infliximab in treating vascular Behçet's disease [52,53]. The largest cohort studied comes from a case report of seven patients with venous, aortic, and retinal involvement. Infliximab initiation resulted in inducing and maintaining remission of vasculitic activity of those patients, with control of inflammation seen 1–5 days after infliximab induction with excellent tolerability [54]. Similarly, in another case report of one patient refractory to treatment with cyclophosphamide and glucocorticoids, initiation of adalimumab resulted in resolution of venous thrombosis and multiple pulmonary artery aneurysms, with no side-effects or relapse being reported [55]. A large cohort study has confirmed the exceptional safety profile of anti-TNF-α and antiinterleukin 1-β agents; out of 85 treatment regimens, during the follow-up all but one were free of any adverse or serious adverse events [56▪]. Very few reports exist concerning the efficacy of anakinra in vascular disease. In one case report, anakinra (150 mg/day) was found to be partially effective in treatment of a patient with multiple venous thrombosis when combined with methotrexate (15 mg/weekly) and colchicine (1 mg/day) [57]. More interestingly, two patients actually developed thrombotic lesions while on treatment with anakinra [57].

Alemtuzumab shows more promising results, where a case study with one patient exhibited some efficacy to both intravenous and subcutaneous alemtuzumab [58]. Alemtuzumab halted active

disease progression, where other medical therapies failed; however, intravenous use led to disease free intervals followed by relapse, whereas the subcutaneous form sustained remission in this patient up till the time of publication [58].

CONCLUSION

Immunosuppressants are currently the mainstay of treatment of vasculo-Behcet. Treatment with biologic agents seems promising, but randomized control studies are needed to improve management strategies. Furthermore, controlled trials are necessary to assess the need to add anticoagulation to the treatment regimen, with emphasis on new oral anticoagulants [56▪].

Acknowledgements

The authors would like to thank Professor Hasan Yazici for helping us in planning this manuscript.

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

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- ▪ of special interest
- ▪▪ of outstanding interest

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The 2018 landscape of RA, PsA, and SpA pathogenesis

Jose U. Scher

2017 has been a very exciting year for our understanding of physiological and pathogenic mechanisms in rheumatic diseases. In this number of Current Rheumatology Reports, a variety of topics are being covered, including advances in bone remodeling, functional genomics, citrullination, and targeted therapies in spondyloarthritis and psoriatic arthritis (PsA).

Adamopoulous outlined a concise and detailed update on how we view inflammatory processes in the context of bone physiology and pathology (pp. 59–64). He emphasizes the notion that bone is not a static tissue, but rather an environment in which constant anabolic and catabolic reactions maintain a homeostatic equilibrium through the coordination of multiple hormones, cytokines growth factors, and cells. However, during a variety of inflammatory processes [i.e., rheumatoid arthritis (RA), PsA, and others] this equilibrium is perturbed, resulting in undesirable modifications that lead to excessive bone loss and, in the case of PsA, simultaneous bone formation [1]. New cellular and immune players are described, particularly those that regulate bone loss in RA and osteoproliferation during fracture repair. Particular attention is devoted to the role of type-17 cells and their signature cytokines [2], osteoclast precursors, osteoblasts, RANKL/OPG, and the potential plasticity of transcription factors, such as NFkB and NFATc1.

Rao and colleagues take on the evolving field of functional genomics, with specific interest in the regulating interactions between tissue resident stroma and immune effector cells. This review delves into state-of-the art technologies and strategies to characterize in ever-evolving detail the phenotype and function of these cells during inflammatory synovio-entheseal disease. The authors bring us summarized up-to-date data on the use of mRNA sequencing, mass cytometry, and fluorescence activated cell-sorting occurring in primary human tissue samples. Of particular importance is the recent discovery of the role and biology of fibroblasts positive for CD90 (Thy1), which are enriched in the synovium of RA patients [3]. This is in the context of the recent discovery by the Rao group of a markedly expanded population of PD-1hiCXCR5−CD4+ T cells in RA synovium [4]. Using multidimensional cytometry, transcriptomics, and functional assays, the group defined, for the first time, this population of 'peripheral helper' T (T_{PH}) cells that also express factors enabling B-cell help, including interleukin (IL)-21, CXCL13, inducible T-cell COStimulator, and macrophage activating factor, ultimately promoting B-cell responses and antibody production within pathologically inflamed nonlymphoid tissues. These examples will likely pave the way for future genomics studies that can lead to the discovery of novel disease mechanisms through the perturbation of molecular pathways, ultimately crating therapeutic targets that are known to affect clinical outcomes (e.g., cytokines, small molecules) [5].

Our understanding of the citrullination process in physiology and RA has expanded significantly over the last decade, even beyond its diagnostic performance in the form of anti-cyclic citrullinated peptides or anti-cyclic citrullinated peptide antibodies (ACPA) measurement. Although the advent of these clinical tools has provided new insights, several aspects of this pathway in relation to inflammatory arthritis remain a matter of debate. Of interest to the field, questions that remain unanswered include whether these autoantibodies against citrullinated neopeptides are indeed proarthritogenic [6], the biological associations of antibody formation with periodontal microbes as triggers for hypercytrullination [7,8], what are the implications of epitope spreading, and what is the true predictive value of ACPAs for the development of future RA. In this issue of current opinion

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Curr Opin Rheumatol 2018, 30:57–58

DOI:10.1097/BOR.0000000000000461

rheumatology, Darrah and Andrade review the latest research on the effects of dysregulated citrullination on driving the production and maintenance of antibodies to citrullinated proteins, a hallmark in RA (pp. 72–78). They describe recent data on mechanisms behind the identity and biological quality of citrullinated peptides in RA synovium, known as 'RA citrullinome'. Of the enzymes capable of citrullination, peptidylarginine deiminases or PADs are the best described and more prominent in RA. A variety of conditions and factors are considered critical regulators of PADs, including calcium influx and redox conditions, occurring both intra- and extracellularly. This was elegantly demonstrated by Damgaard *et al.* providing evidence that PADs are inactivated by oxidation in the extracellular environment [9]. Among pathways known to induce increased intracellular calcium, data points toward the prominent effects of pore-forming proteins (both human and bacterial) leading to PAD activation. The hypercitrullination of the RA joint and the mechanisms behind PAD hyperactivation are discussed in great detail by the authors as well as the potential diagnostic and therapeutic implications of exaggerated anticitrullinated protein immune responses leading to RA in a subgroup of individuals.

Beaten and colleagues offer a granular and clear portrayal of the therapeutic spectrum now available (and on the pipeline) for spondyloarthritis (pp. 79–86). These include tumor necrosis factor blockers, anti-IL17 monoclonal antibodies, JAK inhibitors, and molecules targeting IL-23 [10,11]. The review puts forward the related questions derived from the growing therapeutic possibilities and which strategies are there to optimize the use of these drugs. Multiple aspects of the current challenges in the field include early intervention, combination treatment, and personalized medicine.

In a complementary fashion and as new and effective treatments reach the psoriatic disease market, Soriano's group describes the advances and areas of debate in clinical diagnosis and treatment algorithms in PsA (pp. 87–93). It further reviews some of the novel concepts in disease management, including early diagnosis, remission as an objective, and the concept of treat to target. Comanagement between dermatologists and rheumatologists is also described at length. The controversies surrounding disease activity tools and therapeutic approaches are also discussed at length.

Finally, Amital and colleagues review the latest on the cooccurrence between fibromyalgia syndrome and psychiatric disorders in rheumatic and inflammatory disorders (pp. 94–100). Clinical epidemiology as well underlying immune pathways implicated in pathogenesis.

As the field of inflammatory arthritis progresses toward a wider, ever more detailed understanding of molecular and immune events leading to synovitis and related clinical phenotypes, forward-thinking and potentially paradigm-shifting work is emerging. These include the use of powerful sequencing and flow strategies to characterize potential targets, the study of early events that lead to disease pathology, personalized diagnostic and therapeutic strategies as well as incipient preventive strategies to alter or even prevent the natural course of disease.

Acknowledgements

None.

Financial support and sponsorship

This work was supported by Grant Nos. K23 AR064318 and R03 AR072182 from NIAMS to J.U.S., The Colton Center for Autoimmunity, The Riley Family Foundation, and The Snyder Family Foundation.

Conflicts of interest

J.U.S. has consulted for Novartis, UCB, and Janssen and received honoraria.

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REVIEW

Inflammation in bone physiology and pathology

Iannis E. Adamopoulos[a,b]

Purpose of review
Bone is constantly being remodeled throughout adult life through constant anabolic and catabolic actions that maintain tissue homeostasis. A number of hormones, cytokines growth factors, and the proximity of various cells to bone surfaces influence this process. Inflammatory changes at the bone microenvironment result in alterations leading to both excessive bone loss and bone formation. Detailed understanding of the physiological and pathological mechanisms that dictate these changes will allow us to harness inflammatory signals in bone regeneration.

Recent findings
Recent reports have suggested that inflammatory signals are able to stimulate transcription factors that regulate osteoblast differentiation from their precursors.

Summary
In this review, we summarized current understanding of the roles of inflammation in bone resorption and bone formation, which give rise to different disorders and discuss the huge potential of harnessing these inflammatory signals to achieve bone regeneration.

Keywords
bone loss, inflammation, osteoblasts bone formation, osteoclasts

INTRODUCTION

Tissue regeneration is a biological process appreciated since the Bronze Age, from the ancient Greek myth of Prometheus and his punishment for deceiving the gods and protecting mankind. In the Greek myth, an eagle fed from his liver each day, but the liver regenerated overnight. Although, Prometheus is a mythical creature, liver regeneration in humans is a scientific fact. Indeed epimorphic regeneration occurs in certain animals, such as salamanders and frogs, which are able to regenerate limbs, tails, jaws, and eye lenses. In mammals, deer can regenerate their antlers and mouse their ears. These unusual regenerative properties of certain tissues are a logical adaptation of organisms that are likely to be injured. Specifically, the liver is the main detoxifying organ of the body and is likely to be injured by ingested toxins. Similarly, the skeleton is likely to be fractured which necessitates the ability of fractured bone to regenerate and undergo repair. Apart from fracture healing which is most commonly observed, bone regeneration has also been observed in humans after amputations, especially in children.

Clinical evidence

The ability of bone to regenerate after congenital transdiaphyseal amputation has been recognized since the 1940s and includes numerous case reports of digital phalanx regeneration and a more striking skeletal condition unique to children, known as juvenile amputee overgrowth (JAO). This unique pathological condition is characterized by the growth of a skeletal spike that, in extreme cases, perforates the soft tissue envelope [1]. JAO is certainly no myth and has a prevalence of up to 35% in pediatric amputees and its cellular and molecular mechanisms underlying this phenomenon remain undetermined. A wide range of surgical procedures have been proposed for the management of JAO. Occlusion of the medullary canal appears to significantly retard the progression of JAO and is the current treatment of choice with the best reported results occurring with the use of autogenous bone graft [2,3]. Therefore, current thinking has focused

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Curr Opin Rheumatol 2018, 30:59–64
DOI:10.1097/BOR.0000000000000449

KEY POINTS

- Inflammation regulates bone loss in pathological conditions (rheumatoid arthritis).
- Inflammation regulates bone formation in physiological conditions (fracture repair).
- Inflammation may recruit different precursor cells and pathways than those observed in physiological bone remodeling.

on the progenitor cells and cytokines such as tumour necrosis factor (TNF), Interleukin (IL)-23, IL-17, and Transforming Growth Factorβ that are present within the medullary canal and their interplay under inflammatory conditions with inflamed tissue (Fig. 1). The notion that circulatory cells are able to contribute to bone formation is not observed only in JAO. Interestingly, recent reports suggest that cells with osteogenic potential can be found in a variety of tissues and circulating cells of hematopoietic origin can serve as osteogenic precursors at remote sites of tissue inflammation [4]. This study shows that $CD45^{+}$ hematopoietic-derived circulating osteogenic precursor cells with osteogenic potential migrate to inflammatory sites, where they increase heterotopic bone formation in patients with fibrodysplasia ossificans progressiva (FOP). FOP is an extremely rare autosomal dominant genetic disorder of connective tissue defined by progressive, disabling, heterotopic skeletogenesis in predictable anatomic patterns [5]. Histopathologic analysis of FOP lesions have also revealed an extended inflammatory infiltrate consisting of monocytes and lymphocytes that are commonly associated with pathology and more importantly

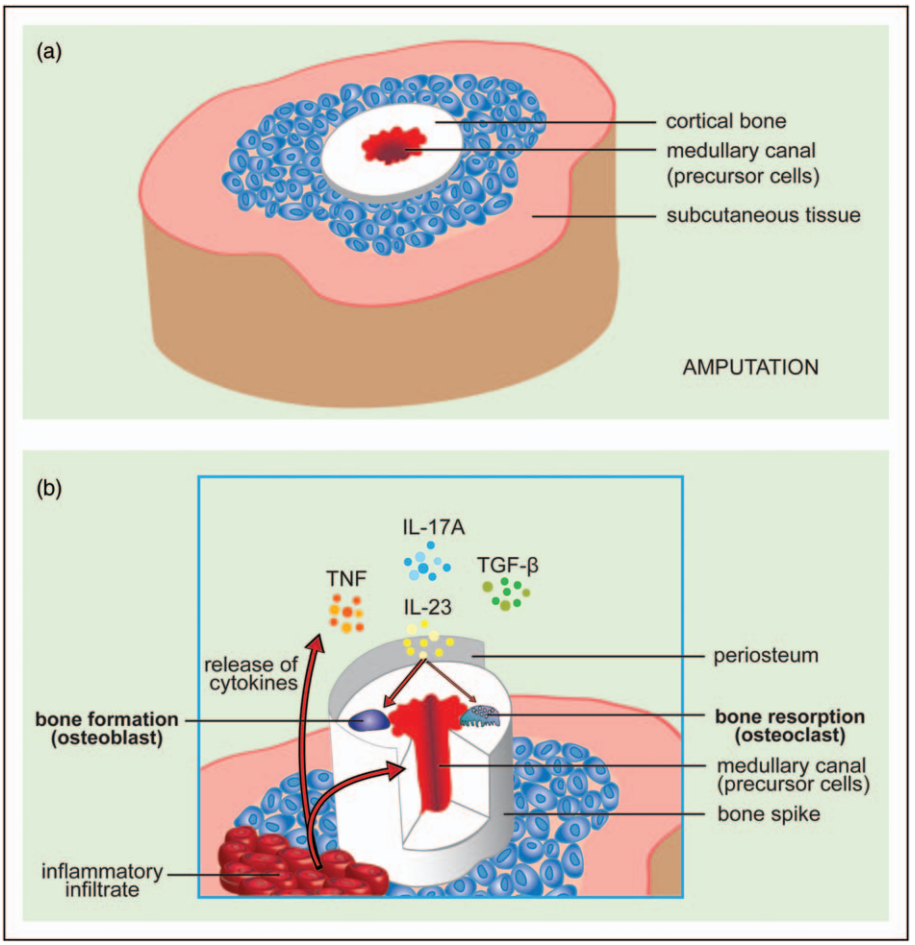

FIGURE 1. Pathophysiology of juvenile amputation overgrowth. Schematic shows the (a) physiological and (b) pathological bone remodeling as it occurs in juvenile amputee overgrowth. Inflammatory infiltrate present after tissue injury (amputation) is responsible for the release of proinflammatory cytokines IL-23 IL-17, TNF, and TGF-β that come into contact with stromal and hematopoietic precursor cells. These molecular changes allow the transcriptional activation of bone cells to promote bone formation causing an abnormal increase in bone growth forming a bone spike. IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor.

accompany spontaneous and trauma-induced exacerbations or flare-ups of FOP [6].

INFLAMMATION IN BONE PHYSIOLOGY AND PATHOLOGY

Increased inflammation in arthritis or after amputation and trauma has always been linked with bone loss via osteoclast activation, and many laboratories have studied this phenomenon extensively in autoimmune diseases but the role of inflammation in bone formation is less clear and not well defined. Approximately, two million people have limb amputations in the United States because of disease or injury with more than 185 000 new amputations every year. The ability to promote epimorphic regeneration, or the regrowth of a biologically based digit or limb, would radically change the prognosis for amputees. However, the significance and impact of understanding molecular pathways that regulate physiological and pathological bone remodeling is far greater and more far-reaching than the amputee populations. In fact, the ability to harness inflammatory signals to regenerate bone may enable the understanding of tissue repair mechanisms that can be applied to a large number of tissues with fundamental benefits.

The osteoclast and the osteoblast are the two main cell types that regulate bone remodeling within the bone. They work in tandem, exhibiting opposing functions; to remove and replace bone, respectively. Under physiological conditions, osteoblasts secrete macrophage colony-stimulating factor (M-CSF) which signals through the tyrosine kinase receptor macrophage colony-stimulating factor receptor (*Csf1r*), which is ubiquitously expressed early during myeloid lineage commitment, and its expression is maintained on nearly all mononuclear phagocytic cells [7]. The differentiation of osteoclast precursors is regulated by the receptor activator of nuclear factor κ-B ligand (RANKL) [8]. RANKL-mediated osteoclast differentiation depends on the RANK receptor, which is upregulated by M-CSF in early stage osteoclast precursors [9]. M-CSF activity generates a specific myeloid subset that expresses both *Csf1r* and *Rank*. This $Csf1r^+/Rank^+$ myeloid precursor is destined to become a terminally differentiated osteoclast on bone surfaces upon stimulation with M-CSF and RANKL. Osteoblasts control the availability of M-CSF and RANKL to osteoclast precursors and also regulate the production and secretion of osteoprotegerin (OPG). OPG is a soluble decoy receptor for RANKL; thus, the main determinants of osteoclastogenesis are the relative concentrations of M-CSF, RANKL, and OPG which regulate a network of gene transcriptions as previously reviewed [10]. The coupling between the osteoblast and the osteoclast at the bone surface, balance bone formation and resorption and maintain bone homeostasis by removing mature bone tissue, following fractures or microfractures, through bone resorption and replacing it with new bone tissue by a process called ossification. The amount of resorption and ossification is, therefore, tightly linked to the number and activity of osteoclasts and osteoblasts and their regulation makes it a necessary event for bone homeostasis and fracture repair [11]. Under inflammatory conditions such as autoimmune diseases, these pathways seem to be disturbed.

Inflammatory bone remodeling

Rheumatoid arthritis is a chronic inflammatory autoimmune disease that exhibits various clinical manifestations, including synovial inflammation and bone loss. Immune cells such as T helper cell17 cells, B cells, macrophages, neutrophils, mast cells, and fibroblast-like synoviocytes are critical for inducing and maintaining synovial inflammation in rheumatoid arthritis pathology [12,13]. This chronic inflammation leads to secretion of a plethora of proinflammatory cytokines and RANKL, which are primarily responsible for the activation of osteoclasts and the subsequent bone destruction.

Transcriptional regulation of bone formation

RANKL regulates a number of transcription factors including Nuclear factor kappa-B (NFkB), which is critical in osteoclast differentiation [10,14]. The Inhibitor of nuclear factor kappa-B kinase (IKK) kinase complex, comprised of two kinases (IKKα and IKKβ) and a regulatory subunit, NF-kappa-B essential modulator/IKKγ is the core element of the NF-κB cascade. This can be activated by RANKL and TNF in both physiological and pathological (inflammatory) conditions to regulate osteoclastogenesis [15]. Interestingly, the same complex seems to have dramatic effects in the regulation of bone formation in osteoblasts [16]. The roles of NFkB transcriptional regulation are diverse and expand in many different cell types that regulate numerous cellular processes. Their functions in bone remodeling include physiological (RANKL) and pathological (TNF)-induced bone loss via osteoclastogenesis and bone formation via the osteoblasts, which have been reviewed elsewhere [17].

Although RANKL is the most potent osteoclastogenic signal in the aforementioned $Csf1r^+/Rank^+$ myeloid populations, osteoclasts have also been generated under inflammatory conditions in

RANK$^{-/-}$ mice [17–19,20▪▪]. In these alternative pathways of osteoclastogenesis that is independent of RANKL, it is clearly evident that a few proinflammatory cytokines including TNF [21,22] and IL-23 [23] regulate the activation of calcium signaling and nuclear factor of activated T-cells cytoplasmic 1 (NFATc1). NFATc1$^{-/-}$ cells are unable to generate osteoclasts, despite normal development into the monocyte/macrophage lineage, highlighting the specific needs of osteoclastogenesis [24]. NFATc1 is a transcription factor activated by calcium signaling, as Ca^{2+} activates calcineurin, which in turn dephosphorylates multiple phosphoserines on NFAT, leading to its nuclear translocation and activation. NFATc1 is responsible for the regulation of genes related to osteoclast function as well as numerous genes nonessential to osteoclast function [25,26]; therefore, the significance of this pathway may extend beyond our current understanding. This is particularly important as other research groups, paradoxically, have observed a role of NFATc1 in bone formation. Although NFATc1 in osteoclasts induces bone loss in osteoblasts, NFATc1 induces bone formation as mice expressing a constitutively nuclear NFATc1 variant (NFATc1nuc) develop high bone mass [27]. Surprisingly, NFATc1nuc mice have massive osteoblast overgrowth and enhanced osteoblast proliferation [27]. Although Runx2/Cbfa1 is considered as the major transcription factor to trigger activation of osteoblast-specific genes [28], at least in one report, NFATc1 regulates bone mass via the osteoblast. The emerging importance of NFATc1 in bone remodeling is because of the fact that Runx2/Cbfa1 is expressed restrictively in osteoblasts, whereas NFATc1 is expressed in both osteoblasts and osteoclasts. More importantly, if indeed that is the case, it seems that NFATc1 transcription factor can regulate bone formation and bone loss in both cell types. This is of particular importance as proinflammatory cytokines such as IL-23 and TNF can induce NFATc1 [22,23].

The notion that inflammation is required for bone regeneration is supported by various observations, most importantly the impaired fracture healing in TNF-deficient mice [29]. The ability of inflammatory signals to induce bone formation is also corroborated by the findings that deregulated bone formation occurs at erosion sites in inflammatory arthritis although it is not clear whether this is a coupling effect where increased bone loss is leading

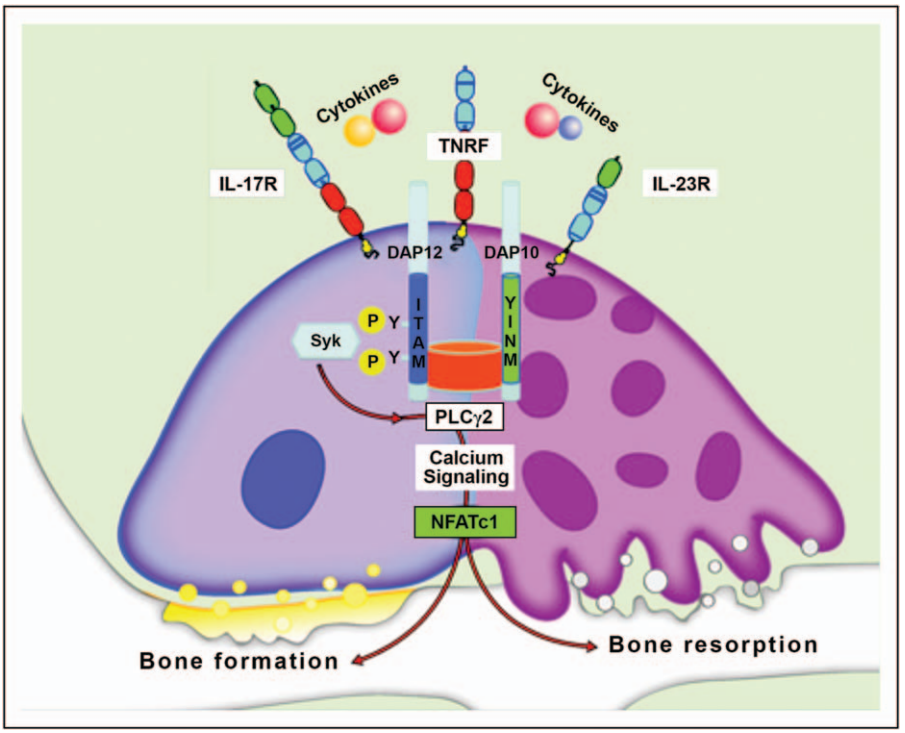

FIGURE 2. Cellular and molecular mechanisms of immune bone loss and bone formation. Schematic representation of proinflammatory cytokine signaling showing activation of NFATc1 through calcium-dependent pathways. Although NFATc1 regulation of genes responsible for both bone loss and bone formation has been reported, certain immunoreceptors are restrictively expressed in myeloid cells. The possibility of cells of hematopoietic origin serving as osteogenic precursors at remote sites of tissue inflammation has also been proposed. NFATc1, nuclear factor of activated T-cells cytoplasmic 1.

to increased bone formation or whether it is a directly inflammation-induced bone formation.

Paradoxical observations

The induction of bone overgrowth in JAO is indeed puzzling and reconciling the paradox of increased bone formation under inflammatory conditions is challenging. The significant overlaps in transcriptional regulation between osteoclasts, osteoblasts, and T cells, by common transcription factors, NFkB and NFATc1 can only partly explain the clinical observations in JAO. This is because although osteoblasts and osteoclasts express TNF and IL-23 receptors the presence of immunoreceptors such as DNAX-activating protein of 12 kda (DAP12) and myeloid DAP12 associated lectin 1 (MDL-1) is critical for NFATc1 stimulation. Indeed, a complex interplay between adaptors and immunoreceptors, including DAP10, DAP12, and MDL-1 can relay activation signals that regulate osteoclastogenesis and bone mineral density [30] (Fig. 2). This is of immense importance as those immunoreceptors are only expressed in hematopoietic cells and not mesenchymal cells. The assumption that circulating cells are of hematopoietic origin, can be manipulated to both bone resorbing and bone forming cells is supported by some evidence [4]. However, before we can explain a paradox with yet another paradox, more experiments in circulating cells need to be performed and a thorough investigation to dissect which cell type responds to inflammation-induced NFkB/NFATc1 activation is much needed. Whether bone formation on hematopoietic precursor cells is possible or not, the certainty is that the molecular mechanisms of inflammatory bone regenerations elude us.

IL-23/IL17 in bone regeneration

Nonconventional γδ T cell receptor $(TCR)^+$ cells are a small (<5% in human peripheral blood) subset of T cells that straddles between the adaptive and innate immune responses and exhibit a high percentage of $IL\text{-}23R^+$ cells (~38%), compared with other innate cells (4–6%) [31,32]. Studies on fracture healing have shown differences in the biomechanical properties of fracture repair, upregulation of cell adhesion molecules in osteoblasts and secretion of inflammatory cytokines at the regenerative site in the absence of γδ TCR^+ cells, suggesting that a more central role of γδ T cells in fracture healing [33]. Other recent studies have specifically shown that $V\gamma6^+$ γδ T cells are the major source of IL-17A in bone regeneration and the action of IL-17 is responsible for enhanced bone formation in a drill-hole injury animal model [34▪▪]. The contribution of IL-23 in the differentiation and expansion of $IL\text{-}23R^+$ T-cell subsets that produce IL-17 to enhance bone formation is also corroborated by other groups. Other groups have also shown that proinflammatory cytokines IL-17 and TNF have the ability to induce gene expression of genes Wingless related integration site 5a, Bone morphogenic protein2, and Runt-related transcription factor2, associated and required for osteoblast differentiation in isolated synoviocytes of arthritic patients [35▪].

CONCLUSION

The role of inflammation has been closely associated with bone loss and osteoclastogenesis, however, it becomes more clear that inflammation has a strong role in bone formation. The same proinflammatory cytokines that have been studied in the context of bone loss in animal models of arthritis are now being studied in bone fracture/repair models. The dual capacities of many transcription factors, including NFkB and NFATc1 that regulate these processes, suggest that adaptor or transducer molecules may fine tune these regulatory elements that need to be identified. Moreover, the specific cell subtypes that give rise to terminally differentiated osteoclasts and osteoblasts need to be better defined [36]. Once we have overcome these obstacles, the exciting prospects of bone regeneration will be realized as a therapeutic approach. For now, although bone regeneration may not be a myth, it certainly merits further investigation.

Acknowledgements

We would like to thank Thanh Nguyen for her assistance with the illustration in the figures.

Financial support and sponsorship

The work was partly supported by NIH/NIAMS-R01AR062173 and the National Psoriasis Foundation Translational Research grant to I.E.A.

Conflicts of interest

There are no conflicts of interest.

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- ▪ of special interest
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Functional genomics of stromal cells in chronic inflammatory diseases

Kamil Slowikowski[a,b,c,d], *Kevin Wei*[e], *Michael B. Brenner*[e], *and Soumya Raychaudhuri*[a,b,c,d,e,f]

Purpose of review
Stroma is a broad term referring to the connective tissue matrix in which other cells reside. It is composed of diverse cell types with functions such as extracellular matrix maintenance, blood and lymph vessel development, and effector cell recruitment. The tissue microenvironment is determined by the molecular characteristics and relative abundances of different stromal cells such as fibroblasts, endothelial cells, pericytes, and mesenchymal precursor cells. Stromal cell heterogeneity is explained by embryonic developmental lineage, stages of differentiation to other cell types, and activation states. Interaction between immune and stromal cell types is critical to wound healing, cancer, and a wide range of inflammatory diseases. Here, we review recent studies of inflammatory diseases that use functional genomics and single-cell technologies to identify and characterize stromal cell types associated with pathogenesis.

Recent findings
High dimensional strategies using mRNA sequencing, mass cytometry, and fluorescence activated cell-sorting with fresh primary tissue samples are producing detailed views of what is happening in diseased tissue in rheumatoid arthritis, inflammatory bowel disease, and cancer. Fibroblasts positive for CD90 (Thy-1) are enriched in the synovium of rheumatoid arthritis patients. Single-cell RNA-seq studies will lead to more discoveries about the stroma in the near future.

Summary
Stromal cells form the microenvironment of inflamed and diseased tissues. Functional genomics is producing an increasingly detailed view of subsets of stromal cells with pathogenic functions in rheumatic diseases and cancer. Future genomics studies will discover disease mechanisms by perturbing molecular pathways with chemokines and therapies known to affect patient outcomes. Functional genomics studies with large sample sizes of patient tissues will identify patient subsets with different disease phenotypes or treatment responses.

Keywords
fibroblasts, functional genomics, inflammation, microenvironment, rheumatic disease, stromal cells

INTRODUCTION

The stroma is the background of every tissue in the body, and plays a critical role in supporting the normal epithelium, forming the general architecture of the tissue, and modulating the local microenvironment. Although many types of cells constitute the stroma in varying proportions across tissues, fibroblasts are the major constituent of stroma in all tissues. One of the first demonstrations of functional fibroblast heterogeneity was done 40 years ago, showing three distinct phenotypes of fibroblast response to prostaglandin [1]. Many studies since then have shown how fibroblasts in different systems support tissue homeostasis and shape immune responses [2].

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Curr Opin Rheumatol 2018, 30:65–71
DOI:10.1097/BOR.0000000000000455

KEY POINTS

- The potential to define disease-related cell types in stroma is rapidly expanding with emerging single-cell technologies such as single-cell RNA-seq and mass cytometry.
- CD90 (Thy-1)-positive synovial fibroblasts are significantly overabundant in rheumatoid arthritis, indicative of major phenotypic changes in the rheumatoid synovium.
- Functional genomics can shed light on the interaction of the stroma with the immune system.

Fibroblasts are central to tissue biology because they organize the extracellular matrix in which other cells are embedded, and they communicate with these cells [2]. Since their functions are dependent on the context in which they are embedded, we face the challenge that in vitro studies may fail to capture physiologically relevant context essential to tissue biology. In contrast to hematopoietic cells, fibroblasts lack well defined cell surface markers that distinguish different functional types of cells subsets. Only one marker, the GPI-anchored protein Thy-1 (CD90), has been studied in many tissues. Thy-1 is differentially expressed on different types of fibroblasts in the spleen [3], lung [4,5], female reproductive system [6,7], ocular orbit [8], liver [9], and prostate cancer tumors [10].

Functional genomics, or discovery of biological insights through genome-wide assays, has revolutionized the way in which we understand cell biology and tissue physiology. By assessing global measures of all genes, it has the potential to find new factors important for production and degradation of extracellular matrix, development of blood and lymph vessels, wound healing, and communication with leukocytes during inflammation.

One limitation of current genomic studies is that they effectively average signals in bulk, high-dimensional assays across millions of cells with diverse phenotypes. This complicates interpretations about the roles of specific cell types. In other words, differences in cell composition cannot be distinguished from differences in gene regulation at the whole tissue level. More recently, studies using low-input genomics on subpopulations sorted by cell surface markers and studies using single-cell genomics demonstrated that the average signals can be explained by independent contributions from different proportions of functionally distinct single cells. Further, single-cell technologies are directly addressing the challenge of discovering cell surface markers that distinguish functional subsets of cells relevant to tissue biology and disease pathology.

FIBROBLASTS MEDIATE INFLAMMATION IN CHRONIC INFLAMMATORY DISEASES

The role of stromal cells in orchestrating local inflammatory response is becoming increasingly appreciated. Recent studies suggest that the diversity of stromal cells across anatomical sites may contribute to location-specific disease development [11▪▪,12▪,13]. There are consistent patterns of anatomical distribution for diseases like RA, inflammatory bowel disease (IBD), psoriasis, ankylosing spondylitis, and various types of cancers. For example, ankylosing spondylitis often affects lower extremities or the spine, whereas RA affects small distal joints of the hands and feet [11▪▪]. These anatomical patterns might be caused by site-specific local cell types, local responses to systemic signals, or environmental factors like mechanical stress that affect cells locally [11▪▪]. For example, one recent study shows that expression of developmental *HOX* genes can influence the tumor necrosis factor (TNF)-induced activation of inflammatory molecular pathways in knee synovial fibroblasts [12▪].

The synovium is a thin membrane composed mostly of fibroblasts and macrophages that surrounds the joint capsule and contains the synovial fluid. In RA, it is the critical site of chronic inflammation [14]. A healthy synovium has a one to two cell-thick lining layer and a sublining layer with blood vessels, lymph vessels, fibroblasts, collagen fibers, nerve fibers, and few leukocytes [15]. In RA, the inflamed synovium has a hyperplastic lining layer, dramatic expansion of blood vessels in the sublining, and dense infiltration by inflammatory leukocytes [15]. Although all of the cells play some role in chronic inflammation, synovial fibroblasts orchestrate joint destruction through production of proteinases that degrade the cartilage and cytokines that activate bone resorbing osteoclasts [16].

Autoimmune diseases, such as RA, progress from an initiation phase of generating autoantibodies to an effector phase of continuous feedback between stromal cells and leukocytes [17,18]. In RA, a vicious cycle of inflammation is fueled by fibroblasts in the synovium. The fibroblasts produce chemokines that recruit leukocytes to the damaged tissue, and these two cell types then continuously stimulate each other to maintain chronic inflammation [19,20]. A recent study shows that there is a similar feedback loop between leukocytes and stromal cells in IBD. In IBD, fibroblasts are activated by TNF and other chemokines, causing them to produce IL6 and other chemokines to activate leukocytes [21▪▪]. In contrast

to the cartilage destruction in RA, the fibroblasts of the gastrointestinal tract are responsible for excessive extracellular matrix deposition, causing fibrosis [22].

As a result of the signaling feedback loops between stromal cells and leukocytes, fibroblasts undergo long-lasting epigenetic changes, become resistant to apoptosis, and maintain a proliferative, destructive, invasive, and migratory phenotype [23–25]. Cultured synovial fibroblasts from inflamed joints of RA patients, but not from healthy joints, express major histocompatibility complex (MHC) class II molecules in response to IFN-gamma [26]. This is consistent with a similar demonstration that fibroblasts within the human lung express human leukocyte antigen – antigen D-related (HLA-DR) and costimulatory molecules, modulating the CD4 T-cell response to pathogens during infection [27]. Hence, many synovial and lung fibroblasts are actually antigen-presenting cells; they are able to extract and present antigens to CD4+ T cells [26–28].

EXPLORING STROMAL CELL BIOLOGY WITH FUNCTIONAL GENOMICS

Functional genomics offers a promising path forward to understand the diversity of fibroblast functions in health and disease. Here we summarize some strategies and glimpse the future of stromal cell genomics (Fig. 1).

Three main strategies are used to apply functional genomics to understand stromal cells in rheumatic disease. First, whole tissue transcriptomics by microarray, RNA-seq, or DNA methylation is used to contrast patients and controls. This type of analysis highlights molecular markers of disease such as cytokines elevated in the disease state, or cell surface markers of differentially abundant cell types. Second, purified cell populations by fluorescence-activated cell sorting (FACS) after tissue dissociation or laser dissection is followed by transcriptomics of selected populations. This strategy reveals the distinct genomic signals underlying the average signals seen in bulk tissues, helping to focus on a smaller set of cell types that are most relevant for disease pathogenesis. Third, the latest single-cell RNA-seq techniques can be performed on mechanically or enzymatically dissociated tissue samples to provide a somewhat unbiased view of thousands of cells in patient samples. This overcomes the need for cell surface markers to sort different cell types, and can give a holistic perspective of how abundances and functions of different cell types relate to each other in patient tissues.

Another important consideration for functional genomics is whether to use cultured cell lines or fresh cells from patient specimens. A major advantage of cultured cell lines derived from patient tissues is that they can be expanded to provide enough RNA, DNA, and other cellular material necessary for most genomic assays. Cell lines, however, lack the context of the whole tissue and are altered by growing *in vitro*. In many instances, studies contrast a small number (<10) of cell lines derived from patients and controls, sometimes including perturbations by therapies or gene knockdowns. A recent study did differential gene expression analysis with microarrays comparing macrophages and fibroblasts from synovial samples from RA and osteoarthritis. The authors found that both cell types are producing inflammatory cytokines in RA [29]. They also identified POSTN and TWIST1 as key regulators of fibroblast invasiveness in RA, and siRNA

Tissue Source	Molecular Assay	Resolution	Study Design
Cell lines Whole tissue • Biopsy • Surgical specimen Phenotypically sorted cells	Genomics • DNA sequencing • Transcriptomics (microarray, RNA-seq, etc.) • Epigenetics (ATAC-seq, ChIP-seq, etc.) Proteomics • Fluorescence-activated cell sorting • Mass cytometry (CyTOF)	Bulk (thousands to millions of cells) Low Input (hundreds of cells) Single-cell	Exploratory (small n) Case control • Disease vs healthy • Disease vs disease • Responder vs nonresponder Case only progression • Early or late disease • Drug response

FIGURE 1. Functional genomics in stromal cells. All studies involve careful considerations of the costs and benefits for selection of a tissue source, molecular assay, desired resolution, and study design. Critically, effective application of high dimensional molecular assays requires effective strategies to disaggregate whole tissue sources without disturbing biological signal.

experiments confirmed this finding [29]. In other study, microarray analysis with osteoarthritis and RA fibroblasts revealed constitutive upregulation of the transforming growth factor beta (TGFB) pathway in RA fibroblasts that resulted in greater expression of MMP11 [30]. Regarding anatomical patterning, genome-wide methylation microarrays and RNA-seq data show differential methylation and expression of developmental *HOX* genes between fibroblasts taken from hips and knees from RA and osteoarthritis individuals [13]. In knee synovial fibroblasts, silencing the *HOTAIR* lncRNA in the *HOX* cluster resulted in greater expression of constitutive and TNF-induced collagenase MMP1 [12▪]. This suggests that the *HOX* genes control stromal cell phenotypes during embryonic development and the immunoregulatory phenotypes during disease pathogenesis.

Functional genomics with fresh cells from patient tissues promises a path toward understanding disease mechanisms *in vivo*. However, fresh human-derived tissue samples are a limited resource, and must be used judiciously. For RA, these samples are often taken from patients in the late stages of disease who elect for joint arthroplasty. One exciting opportunity for querying early stages of RA is the use of research synovial biopsies [31]. Although tissue quantities are limited with biopsies, they enable querying tissues under prescribed clinical conditions, and are possible in a trial setting. In a study using transcriptomics by microarrays, the authors defined a rule set for discriminating RA from osteoarthritis and healthy controls [32]. Another microarray study of RA, osteoarthritis, and normal donors found that RA patients can be divided into two subsets with high or low expression of PRG4 in the synovium. They found that low PRG4 expression is associated with more aggressive stage of disease [33]. Pathogenic fibroblast destruction of cartilage, as well as recruitment and retainment of leukocytes is amplified by hematopoietically derived cytokines such as TNF and interleukin-6 (IL-6). Transcriptomics studies with synovial biopsy tissues revealed that treatment with tocilizumab, methotrexate, adalimumab, and rituximab reduces the mRNA levels of IL-6 and many other chemokines and T-cell activation genes after 12 weeks of therapy [34].

A recent study highlighted the interaction of stromal cells with leukocytes by using functional genomics with tissue biopsies from patients with Crohn's disease and ulcerative colitis. In that study, West *et al.* [21▪▪] found a TNFi-resistant pathway specific to gut-resident stromal cells. Analysis of public transcriptomics data put the focus on oncostatin M (OSM) because it is more highly expressed in biopsy tissues from IBD patients. Next, the authors found that OSM is mostly derived from hematopoietic cells, and stromal fibroblasts have highest expression of the OSM receptor (OSMR) in the gut. These PDPN+ fibroblasts are highly abundant in the inflamed tissue from Crohn's disease and ulcerative colitis patients, and they produce inflammatory chemokines in response to TNF and OSM. This study highlights the novel system of leukocyte–stromal communication behind the chronic inflammation in IBD.

The OSM result may translate to human therapies. Notably, phase-1 and phase-2 trials for RA show that humanized anti-OSM monoclonal antibodies are well tolerated, and OSMR-deficient mice are healthy and viable. Additionally, large scale studies with functional genomics on many biopsies can help to identify different subsets of patients. One such study had 210 patients with Crohn's disease and 35 healthy controls. This study found that patients with Crohn's disease were clearly distinguished from healthy controls by biopsy gene expression of 29 genes enriched with genetic risk variants [35]. Further, the 29-gene signal was also able to identify which patients were more likely to progress to complicated disease. Taken together, functional genomics in stromal cells reveals how stromal cell interact with leukocytes, and it helps to define clinically relevant patient subsets in RA and Crohn's disease.

OPPORTUNITIES TO ADVANCE FIBROBLAST BIOLOGY WITH SINGLE-CELL GENOMICS

Transcriptomic profiling of single cells is a powerful technique to reveal cellular and molecular heterogeneity in tissues with complex and dynamic cellular compositions. These technologies are being applied at scale in efforts such as the human single-cell atlas, with the goal of providing a reference map of all common types of cells in the human body [36]. These types of large-scale studies will transform our understanding of cell identity and function. Single-cell RNA-seq can sometimes overcome the need for cell surface markers to sort different cell types, and can give a holistic perspective of how abundances and functions of different cell types relate to each other in patient tissues.

Recent advances in immunoprofiling and transcriptomic assay technologies has created an opportunity to assay sorted stromal cells directly from tissues [37]. Advances in sequencing library construction have lowered the requirements for genomics assays to tens or hundreds of cells per sample [38]. To apply single cell technologies successfully, it

is critical to carefully dissociate tissue into a single-cell suspension and preserve cell viability. Recently, advances in droplet-based platforms for single-cell RNA-seq have enabled analysis of large number of single cells from a single donor [41,42].

Single-cell genomics will include discover new cell types specific to anatomical sites or disease states. It will also reveal dysregulations of molecular pathways exclusive to particular cell types, and genetic effects on those pathways [39]. One recent study demonstrated that genetic effects on gene expression can be masked whenever assaying total peripheral blood mononuclear cells (PBMCs). This single-cell expression quantitative trait loci (eQTL) analysis showed that the same variant influences expression of TSPAN13 in CD4 T cells, but not in dendritic cells or other cell types [40].

Investigators have begun applying single-cell technologies to query tissues in pathological conditions.

Two independent studies have identified Thy-1-positive and Thy-1-negative fibroblasts in the synovium [41,43]. By applying cell sorting, low-input RNA-seq, and single-cell RNA-seq to synovial biopsies, they identified different functional subsets of fibroblasts [43]. Notably, the Thy-1 positive fibroblasts were found to be significantly overabundant in the synovial tissue of RA relative to osteoarthritis [43]. We checked if we could identify overabundance of Thy-1-positive fibroblasts in a recent study of synovial biopsies [44] and found that RA has higher relative expression of Thy-1-positive fibroblast genes than Thy-1-negative fibroblast genes (Fig. 2). Expression of Thy-1 influences multiple aspects of fibroblast biology such as propensity to differentiate into adipocytes [45], and this protein is also involved in neuronal development and oncogenesis [5].

Despite these exciting initial applications, little is known about the complexity of stromal populations, and single-cell genomics offers a powerful way to capture the heterogeneity and compare gene expression of membrane proteins, transcriptional regulators, and signaling pathways that might reveal phenotypic and functional subsets of fibroblasts. Building on the foundation of the human single-cell atlas, new studies will focus on relevant tissues from patients. In the coming years, each new study of single cells from chronic inflammatory diseases will give deeper insights about the commonalities and differences between them. For example, the Accelerating Medicines Partnership (AMP) consortium is focused on generating large scale datasets that include single-cell genomics from disease-affected tissues in RA and systemic lupus erythematosus (SLE) patients. These datasets will show how cell-type abundances and functions change over the course of disease and in response to therapy. We envision that many future studies will take advantage of the new clarity provided by single-cell technologies.

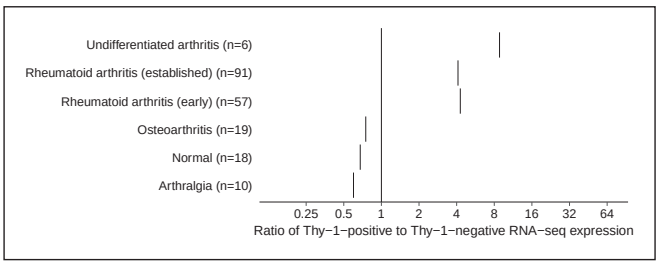

FIGURE 2. Thy-1-positive synovial fibroblast gene expression in RA. First, we selected four genes from single-cell RNA-seq data [43] that are highly expressed in sublining CD34-negative Thy-1-positive fibroblasts (*THY1*, *POSTN*, *TGFBI*, *CCL2*) and four genes highly expressed in lining CD34-negative Thy-1-negative fibroblasts (*CLIC5*, *TSPAN7*, *SLC2A12*, *SELP*). Next, we took the synovial biopsy RNA-seq expression data from a recent study (GSE89408), converted counts to counts per million (CPM), computed the sum of each gene set, and plotted the ratio of the gene sets. Vertical lines are medians. Early RA is significantly different than osteoarthritis (Wilcoxon rank sum test) ($P = 8.5 \times 10^{-4}$), and established RA is also significantly different than osteoarthritis ($P = 9.7 \times 10^{-5}$). This result is consistent with the recently reported results that synovial biopsies from RA tissues have an overabundance of the Thy-1-positive fibroblast population relative to tissue from normal or osteoarthritic patients.

CONCLUSION

In the past few years, oncologists have moved from general chemotherapy to personalized treatment based on genetic measurements. Treatment of rheumatic disease can follow a similar path as ongoing and future studies pave the way forward [46]. For example, measurement of the novel OSM gene expression biomarker in IBD patients may predict successful response to anti-TNF therapy [21▪▪]. As synovial biopsy tissue acquisition becomes more common, researchers will have more opportunities to use functional genomic technologies to uncover disease-relevant molecular phenotypes. Researchers will assay transcriptomics, DNA methylation, chromatin accessibility, histone modifications, and other molecular signals to find associations with disease activity and response to therapies. Armed with this information, biopsy data may be then used to predict optimal treatments for patients. The advancement of molecular phenotyping will help to deliver on the promise of precision medicine, wherever therapies are adjusted for maximum benefit to each individual patient.

Functional genomics has the potential to reveal common and differential sets of molecular pathways across multiple anatomical sites and diseases. Stromal biology has thus far been mostly focused on single systems like cancer-associated fibroblasts from prostate cancer, gut fibroblasts from IBD patients, or synovial fibroblasts from inflamed joints. Many pathological perturbations of stromal cells are likely to be different across diseases. However, careful analysis of the genome-wide measurements from functional genomic techniques can reveal the commonalities in molecular phenotypes across those diseases. For example, the cytokine gene expression profiles of prostate cancer-associated fibroblasts are similar to synovial fibroblasts from RA joints as well as fibroblasts from the gut.

We envision that synovial biopsy samples will be treated *in vitro* to study responses to known and novel therapies. We expect that hematopoietic and stromal cells from treatment responders might behave differently than cells from nonresponders, and future studies will find the molecular basis for these differences. These findings will give unprecedented resolution about pathogenic functions in the synovium, and will suggest new diagnostic assays and therapeutic targets that might be addressed by existing or future therapies [15].

Acknowledgements

We are very thankful to Ilya Korsunsky for valuable discussions.

Financial support and sponsorship

This work is supported in part by funding from the Ruth L. Kirschstein National Research Service Award (F31AR070582) from the National Institute of Arthritis and Musculoskeletal and Skin Diseases (K.S.), the Rheumatology Research Foundation Scientist Development Award (K.W.), the National Institutes of Health (UH2AR067677, 1U01HG009088, and 1R01AR063759), and the Doris Duke Charitable Foundation Grant #2013097 (S.R.).

Conflicts of interest

There are no conflicts of interest.

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Rheumatoid arthritis and citrullination

Erika Darrah and Felipe Andrade

Purpose of review
Dysregulated citrullination is a key element that drives the production and maintenance of antibodies to citrullinated proteins, a hallmark in rheumatoid arthritis (RA). This article reviews recent literature on the origin of citrullinated antigens in RA.

Recent findings
The study of synovial fluid from patients with RA has provided important insights into the identity of citrullinated proteins that accumulate in the RA joint (the RA citrullinome) and mechanisms that control their generation.

Summary
Citrullinating enzymes (peptidylarginine deiminases, PADs) are tightly controlled to limit their hyperactivation. Calcium and redox conditions are important regulators of PAD activity. Studies suggest that citrullination is dysregulated both intra- and extracellularly in RA. In neutrophils, host (i.e., perforin and the membrane attack complex) and bacterial (i.e., toxins) pore-forming proteins induce prominent calcium influx, cytolysis, and hyperactivation of PADs. These factors likely drive hypercitrullination in the RA joint and at extraarticular sites of disease initiation, respectively. As oxidizing conditions present in the extracellular environment are known to inactivate PADs, extracellular citrullination in RA probably requires the constant release of active enzymes from dying cells and may be accelerated by autoantibodies that activate PADs.

Keywords
anticitrullinated protein antibody, citrullination, citrullinome, leukotoxic hypercitrullination, peptidylarginine deiminase, rheumatoid arthritis

INTRODUCTION

The nonessential amino acid citrulline was isolated from the juice of the watermelon, *Citrullus vulgaris*, by Koga and Ohtake in 1914 [1]. Wada established the structure of citrulline in 1930 and provided the first evidence that this amino acid can be found in proteins [2,3]. Further studies by Rogers *et al.* demonstrated that citrulline was enzymatically generated by side-chain conversion of peptidylarginine to peptidylcitrulline in a calcium-dependent process known as deimination or citrullination [4–6]. The enzyme responsible for this reaction was partially purified by Fujisaki and Sugawara in 1981 and named peptidylarginine deiminase (PAD) [7]. Five PADs have since been identified in humans (PAD1–4 and 6) [8–13] and are located in a cluster on chromosome 1p36.1 [12]. While the discovery of citrullination generated interest in different areas of research, the finding by Schellekens *et al.* that citrullinated proteins are major targets of antibodies in patients with rheumatoid arthritis (RA) [14] sparked major interest in understanding the role of citrullination in RA pathogenesis. Here, we discuss the most recent evidence regarding the causes and significance of dysregulated production of citrullinated proteins in RA, collectively referred to as the RA citrullinome.

MECHANISMS THAT REGULATE PHYSIOLOGIC CITRULLINATION

In order to discuss pathways that may lead to dysregulated protein citrullination in RA, it is important to first review what is known about the physiologic mechanisms regulating protein citrullination and the significant gaps in knowledge that

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Curr Opin Rheumatol 2018, 30:72–78
DOI:10.1097/BOR.0000000000000452

KEY POINTS

- The RA citrullinome comprises a unique set of intra- and extracellular citrullinated proteins that are highly enriched in the rheumatoid joint. The abnormal accumulation of citrullinated proteins suggests that mechanisms controlling citrullination are dysregulated in RA.
- Calcium and reducing conditions are necessary for efficient PAD activation. Oxidizing environments inhibit PAD activity.
- Pathways that promote suprathreshold amounts of intracellular calcium, such as host (i.e., perforin and membrane attack complex) and bacterial (i.e., toxins) pore-forming proteins, are potent activators of PADs and inducers of hypercitrullination.
- Bacterial and host pore-forming pathways likely sustain citrullinated autoantigen production at extraarticular sites of disease initiation and in the RA joint, respectively.
- As PADs are oxidized and inactivated extracellularly, efficient extracellular citrullination in RA likely requires PAD activating cofactors (e.g. anti-PAD activating antibodies) and the continual release of active PAD enzymes from dying and activated cells.

still exist. In contrast to many other posttranslational modifications (PTM), citrullination appears to be an irreversible process. 'De-citrullinating' enzymes that convert citrullinated proteins back to their native peptidylarginine containing forms have not been discovered. The mechanisms involved in the clearance or turnover of citrullinated proteins in cells also remain unknown. As citrullination reduces the net charge of proteins through the loss of one positive charge per modified arginine residue, it can increase protein hydrophobicity, lead to protein unfolding, and alter intra- and intermolecular interactions [15]. These structural changes can lead to either gain or more likely, loss of protein function [15–21]. Given that citrullination can have important and potentially irreversible consequences on protein function, the role of citrullination as a mechanism to control cellular processes must be tightly regulated to avoid excessive citrullination of physiologic targets or citrullination of nonphysiologic substrates.

One component that controls PAD activation is calcium [6,7]. Binding to calcium induces conformational changes that generate the active form of the enzymes [22]. While full activation of PADs requires millimolar amounts of calcium *in vitro* [7,23], PAD activation in cells is observed under physiologic conditions where intracellular calcium does not exceed nanomolar concentrations [19,24–27]. It is possible that these suboptimal calcium concentrations induce a PAD conformation that selects for only high efficiency substrates, thereby limiting aberrant citrullination events. Additionally, it is suspected that intracellular protein cofactors may be responsible for modulating calcium sensitivity and specificity of the PAD enzymes, but such binding partners have not been identified. Considering the importance of calcium in PAD activation, pathways that dysregulate calcium binding to PADs (as discussed below) are likely relevant to RA pathogenesis.

Another important component for efficient PAD activity is the presence of a reducing environment [6,7], which is necessary to maintain the active site free thiol cysteine required for catalysis [28]. A recent study found that synovial fluid is unable to sustain PAD activity unless reducing agents, such as dithiothreitol or reduced glutathione, were present [29]. The oxidizing nature of the extracellular environment, which contrasts with the reducing environment inside cells [30], may therefore provide the conditions needed to protect against aberrant extracellular citrullination by PADs that may leak from activated or dying cells. The importance of reactive oxygen species (ROS) in controlling PAD activity has been recently underscored by the finding that ROS generated by NADPH oxidase inhibits the catalytic activity of PAD2 and PAD4 [31▪▪]. The activation of NADPH oxidase as a mechanism to limit PAD activation may play an important role in preventing hypercitrullination during neutrophil extracellular trap (NET) formation [32], in which citrullination may have deleterious effects on the antimicrobial activity of NETs [33▪▪].

DEFINING THE RHEUMATOID ARTHRITIS CITRULLINOME

A key sustaining component in autoimmune rheumatic diseases is the autoantigen, which fuels the ongoing immune response. Synovial fluid from patients with RA contains a unique pattern of citrullination that includes proteins spanning the range of molecular weights, termed hypercitrullination [34]. Proteomic analysis of the cellular and soluble components in RA synovial fluid have identified more than 100 citrullinated proteins [35–37], which include both intra- and extracellular substrates and together comprises RA citrullinome. The significance of this citrullinome to RA pathogenesis and the generation of anticitrullinated protein antibodies (ACPAs), however, are not fully understood.

Despite the considerable number of citrullinated proteins found in RA synovial fluid, only few have been identified as being targets of ACPAs (e.g., vimentin, α-enolase, and fibrinogen). Whether unique ACPAs exist for each protein in the RA citrullinome or whether only few citrullinated proteins drive the complete ACPA response is still unknown. Given that citrullination is a physiologic process, it is unclear why this PTM becomes a target of an abnormal immune response in RA.

Interestingly, while citrullination is clearly an important process for the physiologic function of proteins, such as trichohyalin, filaggrin, histones, and transcription factors [6,24,26,27,38–45], it is unknown whether the majority of proteins that comprises the RA citrullinome are physiologic or accidental pathologic targets of PADs. In the case of well-defined citrullinated autoantigens like α-enolase and vimentin, for example, hypercitrullination *in vitro* inactivates their function [16,20]. However, it is unknown whether this modification is part of the normal regulation of these proteins or only occurs in pathologic conditions.

If nonselective citrullination occurs accidentally as a consequence of uncontrolled PAD activation, it may lead to the generation of neocitrullinated proteins not previously tolerized by the immune system and consequently trigger an autoimmune response in susceptible individuals. Alternatively, it is possible that hyperactivation of PADs may accidentally target novel sites in proteins, generating nontolerized neoepitopes in abnormally hypercitrullinated molecules. In this regard, recent work demonstrated that while fibrinogen is more extensively citrullinated by PAD2 compared with PAD4 [46], distinct partially citrullinated forms of fibrinogen induced by PAD4 are preferentially recognized by ACPAs [47▪]. This supports the hypothesis that the generation of unique citrullination sites in proteins may drive their immunogenicity. The production of neocitrullinated proteins or epitopes may occur and propagate specifically in RA target tissues, such as the joints, due to the establishment of amplification cycles in which damage to PAD-expressing cells by immune components leads to further hypercitrullination (discussed below). This may explain why other tissues highly enriched with physiologically citrullinated proteins, such as the skin, are not pathologic targets for ACPAs.

THE ORIGIN OF THE RHEUMATOID ARTHRITIS CITRULLINOME

The RA citrullinome is comprised of intra- and extracellular proteins, suggesting that PADs are dysregulated in both compartments. Any PAD-expressing cell present in the RA joint could contribute to the RA citrullinome, including immune cells and resident fibroblast-like synoviocytes (FLSs) [48,49]. Recent studies have revealed neutrophils, the most abundant immune cells in RA synovial fluid [50], as major sources of intracellular citrullination and soluble PADs for extracellular citrullination [34,51,52▪▪,53]. FLSs and monocytes have also been shown to generate citrullinated α-enolase and vimentin, known RA autoantigens, following treatment with specific stimuli [54,55]. Mechanisms implicated in generating the RA citrullinome will be discussed in detail below.

MEMBRANOLYTIC PATHWAYS AS DRIVERS OF THE INTRACELLULAR RHEUMATOID ARTHRITIS CITRULLINOME

The finding that cells in RA synovial fluid have marked citrullination has focused attention on understanding mechanisms of hypercitrullination that can reproduce the RA citrullinome. Analysis of a broad range of stimuli that induce neutrophil activation and death identified that perforin and the membrane attack complex (MAC), two immune-mediated membranolytic pathways, have the unique capacity to reproduce similar patterns of hypercitrullination observed in RA synovial fluid [34]. Perforin and MAC are pore-forming cytolytic proteins that induced the influx of ions, particularly calcium, and osmotic lysis [56–58]. The form of cell death induced in neutrophils by these pore-forming mechanisms has recently been named leukotoxic hypercitrullination (LTH), to distinguish it from other forms of neutrophil death that do not induce hypercitrullination [33▪▪]. However, even nonlethal amounts of perforin and MAC can lead to significant increases of intracellular calcium to the micromolar range [59,60], so may also support hypercitrullination. The abrupt and prominent influx of calcium resulting from pore-induced membranolytic damage likely overcomes regulatory pathways that control PAD activation in cells, leading to hyperactivation of the enzymes and subsequent hypercitrullination.

The ability to trigger calcium flux-induced LTH is not only limited to host immune pore-forming pathways, but also includes bacterial products such as calcium ionophores (i.e., ionomycin and calcimycin from *Streptomyces* species) and pore-forming toxins [33▪▪,52▪▪]. In this regard, the tantalizing idea that periodontal disease may initiate RA has been recently strengthened by the finding that the periodontal pathogen *Aggregatibacter actinomycetemcomitans* activates cellular hypercitrullination in neutrophils [52▪▪] via secretion of the pore-forming

protein leukotoxin A (LtxA). Like host pore-forming proteins, LtxA induces target cell death by membrane destabilization, influx of extracellular calcium, and osmotic lysis [61,62]. Importantly, both the citrullinome induced by LtxA and the citrullinome present in the periodontium in periodontal disease have high similarity to the citrullinome found RA synovial fluid [52▪▪]. Remarkably, the association of ACPAs with human leukocyte antigen-DRB1 alleles linked to RA appears to be significant only in patients with RA that had evidence of a history of infection with leukotoxic strains of *A. actinomycetemcomitans* [52▪▪], as measured by the presence of antibodies to LtxA. This suggests that *A. actinomycetemcomitans* may play a role in ACPA development in individuals with a genetic predisposition to develop RA.

Interestingly, bacteria that colonize and infect other mucosal surfaces implicated in RA, such as the lung, gut, and urothelium [63,64], also rely on the production of pore-forming toxins as virulence factors to target neutrophils [65–67]. Initial characterization of some of these toxins has confirmed that other pathogenic bacteria, such as *Staphylococcus aureus* (via Panton–Valentine leukocidin), also have the capacity to induce neutrophil hypercitrullination [33▪▪]. This suggests that membranolytic damage induced by several different bacterial pathobionts may stimulate chronic hypercitrullination in neutrophils, ACPA production, and RA development in susceptible individuals. Once an ACPA response is initiated at an extraarticular site against bacterial toxin-induced hypercitrullination, host pore-forming pathways (i.e., perforin and MAC) may be responsible for sustaining antigen production in the RA joints. In this scenario, bacteria may be necessary for disease initiation, but may not be required to sustain ongoing immune-mediated damage in the articular compartment.

CELL DEATH PATHWAYS AS DRIVERS OF THE INTRA- AND EXTRACELLULAR RHEUMATOID ARTHRITIS CITRULLINOME

Several mechanisms of cell death have been implicated in generating citrullinated autoantigens in RA, including autophagy, NETosis, necrosis, and more recently LTH (discussed in detail above). These forms of cell death could induce intracellular PAD activation and de-novo citrullinated protein generation, as well as the release of transiently active PADs into the extracellular environment [33▪▪,53,54,68]. NETosis has gained attention as a mechanism to generate and release citrullinated autoantigens extracellularly, thereby triggering ACPA-associated experimental arthritis [68,69]. Nevertheless, recent evidence has demonstrated that citrullination is not required for the formation of NETs (at least in human neutrophils) [32,70,71,72], bringing into question the role of NETs in the abnormal production of intracellular citrullinated autoantigens in RA. While some citrullinated proteins can be detected in NETs by mass spectrometry [53,69], it is noteworthy that several of these proteins (such as actin, histones, actin-related protein 2/3 complex subunit 1B, coronin, and leukocyte elastase inhibitor, among others) [53,69] are also citrullinated in unstimulated neutrophils [34]. This raises the possibility that NETosis is only a redistributor of a limited steady state citrullinome in neutrophils, rather than a generator of de-novo pathogenic citrullination in RA.

Although autophagy does not induce hypercitrullination in neutrophils [34], a recent study demonstrated that this programmed cell death mechanism does lead to the citrullination of α-enolase and vimentin in monocytes and FLSs [54]. These citrullinated antigens are known targets of ACPAs and increased autophagy markers were reported to correlate with ACPA titers, suggesting that autophagy may contribute to generation of citrullinated proteins in patients with RA [54].

While many forms of cell death may be responsible for releasing PADs extracellularly in RA, only NETosis and necrosis in neutrophils have been studied in detail [53]. Spontaneous release of nuclear material, described as NETosis, has been reported to occur in neutrophils from patients with RA, resulting in the extracellular release of PAD4 [73], and increased nuclear material and PAD activity is present in the synovial fluid from patients with RA compared with osteoarthritis [53]. In in-vitro studies measuring extracellular PAD activity following cell death, significantly more active PAD enzyme was released following necrosis, compared with NETosis [53]. While the relative contributions of NETosis, necrosis, and other forms of cell death to extracellular PAD enzyme release in RA are unknown, these findings indicate that the release of extracellular PADs in RA is not representative of a single form of cell death.

AUTOANTIBODIES AS DRIVERS OF THE EXTRACELLULAR CITRULLINOME IN RHEUMATOID ARTHRITIS

Irrespective of the mechanism, increased soluble extracellular PAD2 and PAD4 is observed in the synovial fluid from patients with RA [53,74]. Once released, these PADs have the potential to citrullinate extracellular substrates and interact with anti-PAD autoantibodies. Autoantibodies that enhance the catalytic activity of PAD4 by lowering the amount of calcium required for catalysis were

recently described in RA [75]. These antibodies distinguish a unique subgroup of patients with the most erosive disease and pulmonary involvement [75–77], supporting their potentially pathogenic role in RA. Interestingly, a recent study found that the oxidative environment in synovial fluid is unable to sustain PAD activity [29]. As PADs released from activated and dying neutrophils are transiently active [29], it is possible that PAD4-activating antibodies may act by increasing PAD function despite the non-permissive conditions in synovial fluid, thus enhancing dysregulated extracellular citrullination. Defining the citrullinated proteins generated in the presence of PAD4-activating antibodies will be important for understanding their contribution to the RA citrullinome.

CONCLUSION

The prominent accumulation of intra- and extracellular citrullinated proteins in the rheumatoid joint strongly suggests that citrullination is dysregulated in RA (Fig. 1). The experimental replication of the cellular RA citrullinome requires stimuli that

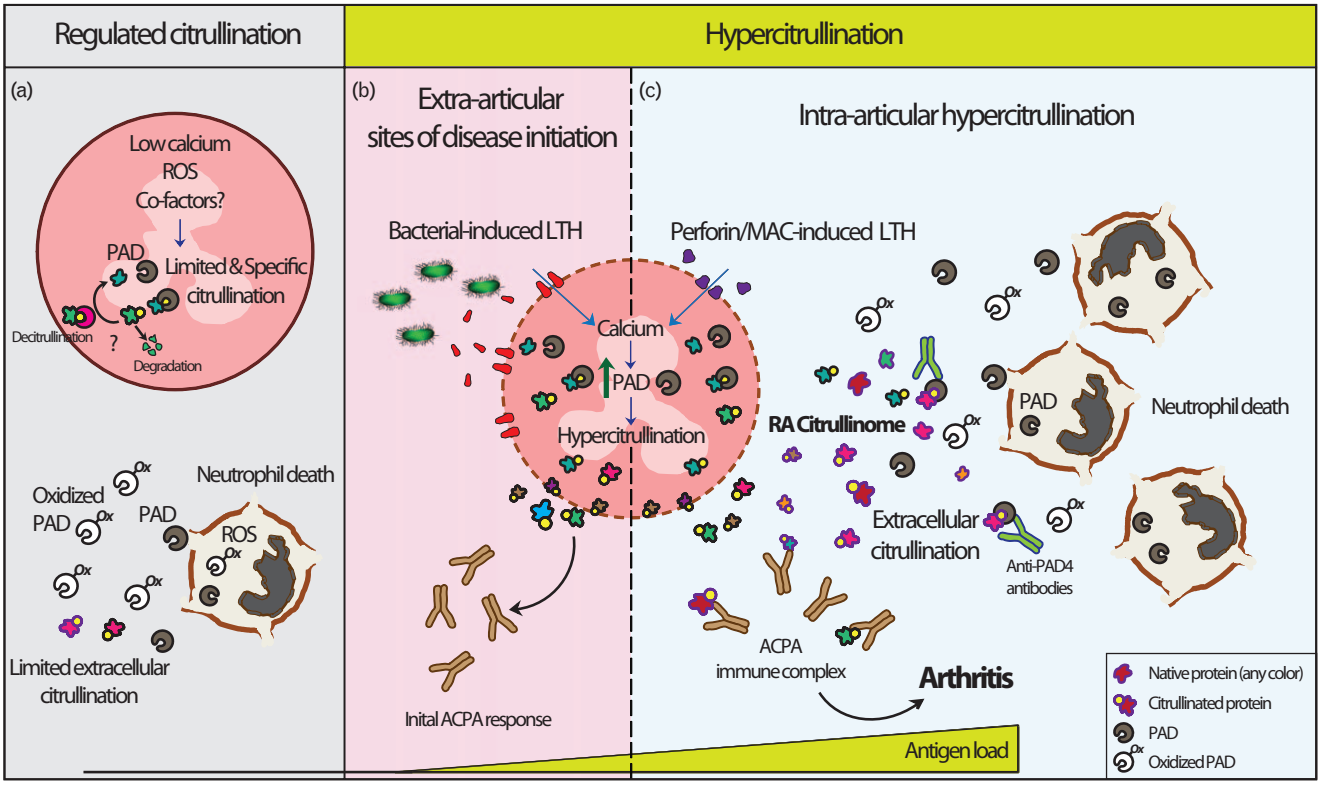

FIGURE 1. Normal and dysregulated citrullination in rheumatoid arthritis (RA). (a) Peptidylarginine deiminase (PAD) function is limited by transient nanomolar fluctuations in intracellular calcium, oxidizing environments, and potentially protein cofactors. By maintaining a suboptimal activity of PADs, these components may increase enzyme specificity, avoiding the abnormal citrullination of nonphysiological substrates. Moreover, efficient mechanisms of clearance of citrullinated proteins (likely by degradation and less likely by reconversion of citrulline residues to arginine residues) are important to prevent their abnormal accumulation in cells. During physiologic forms of cell death, such as neutrophil extracellular trap formation, PADs are likely inhibited by reactive oxygen species to prevent hyperactivation as result of calcium influx in dying cells. Similarly, oxidation appears to protect against extracellular citrullination by PADs released from activated and dying cells. Through regulated citrullination, the load of immunogenic proteins is insufficient to drive an anticitrullinated protein antibody (ACPA) response under physiologic conditions. (b, c) Hypercitrullination results from mechanisms that overactivate the PAD enzymes. Membranolytic damage induced by host and bacterial pore-forming proteins are potent inducers of leukotoxic hypercitrullination (LTH). (b) Bacterial pore-forming toxins are potential triggers of LTH and ACPA production in extraarticular sites of diseases initiation (e.g., gums, gut, lungs, and others). (c) Immune-mediated membranolytic pathways, such as perforin and membrane attack complex, likely sustain hypercitrullination in the rheumatoid joint. The large number of dying neutrophils in the articular compartment in RA likely maintains a constant release of active PADs for extracellular citrullination. The presence of agonistic antibodies to PADs may enhance extracellular citrullination before the enzymes are inactivated by oxidation. Together, citrullinated proteins from intra- and extracellular sources constitute the RA citrullinome. Uncontrolled hypercitrullination generates suprathreshold amounts of nontolerized antigens that may initiate an ACPA response and RA in genetically susceptible individuals.

produce damage to the cell membrane, a prominent increase in intracellular calcium concentrations, and cytolysis (e.g., LTH). As such, pathobionts producing pore-forming proteins may be important in generating nontolerized neocitrullinated epitopes at extraarticular sites of disease initiation, including the mouth, gut, lungs and others (Fig. 1B). Once tolerance is broken to the citrullinated products of bacterial-induced hypercitrullination, host pore-forming proteins can sustain ongoing citrullinated autoantigen generation in the joints of patients with RA (Fig. 1C). The production of the extracellular RA citrullinome likely requires a constant release of PADs from activated and dying cells, as well as the presence of cofactors that increase PAD activity (such as autoantibodies) to maintain efficient citrullination in the inhospitable oxidizing extracellular environment. Thus, inhibiting pathways that lead to PAD enzyme hyperactivation could suppress ongoing generation of the RA citrullinome and provide therapeutic benefit to patients with RA.

Acknowledgements

None.

Financial support and sponsorship

The work was supported by the Rheumatology Research Foundation, Jerome L. Greene Foundation, Ira T. Fine Discovery Fund, Department of Defence (DoD) office of the Congressionally Directed Medical Research Programs (CDMRP) grant number W81XWH-15-1-0159, National Institute of Arthritis, and Musculoskeletal and Skin Diseases (NIAMS)/National Institutes of Health (NIH) grant R01 AR069569. The content is solely the responsibility of the authors and does not necessarily represent the official views of NIAMS or the NIH.

Conflicts of interest

E.D. and F.A. are authors on issued patent no. 8 975 033, entitled 'Human autoantibodies specific for pad3 which are cross-reactive with pad4 and their use in the diagnosis and treatment of rheumatoid arthritis and related diseases.' E.D. previously served on the scientific advisory board for Padlock Therapeutics, Inc. F.A. and E.D. received a grant from Medimmune. F.A. serves as consultant for Bristol-Myers Squibb.

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New treatment paradigms in spondyloarthritis

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Purpose of review
The review presents the recent rapid expansion of therapeutical options in spondyloarthritis. Additionally, it focuses on the importance of additional questions raised by the growing therapeutic possibilities related to the optimal use of these drugs.

Recent findings
The emergence of new treatment options opens new avenues and opportunities for treating patients with nonresponse, contraindications, or intolerance for classic drugs. However, it becomes more relevant than ever to define not only drugs and treatment options but also treatment strategies. We address current literature and remaining questions on strategies such as early intervention, combination treatment, personalized medicine, and treat-to-target.

Summary
Not only the treatment as such, but also the treatment strategy is crucial to reveal the full therapeutic potential and benefit for patients. Whereas cautious but crucial steps have been taken in the last years to explore these aspects, related to timing and sequence of treatment (including combination treatments), stratified medicine approaches, and treat-to-target strategies, it is now time for full-scale investment in prospective strategy trials.

Keywords
spondyloarthropathy, strategy trials, treatment

INTRODUCTION

Spondyloarthritis (SpA) is an inflammatory musculoskeletal diseases comprising different phenotypic subsets with common genetic, radiologic, and clinical features [1,2]. SpA is subdivided clinically in axial SpA (AxSpA) and peripheral SpA (pSpA), with ankylosing spondylitis (AS) and psoriatic arthritis (PsA) as clinical prototypes, respectively [3]. The key features of AxSpA are inflammatory back pain, sacroiliitis, and new bone formation leading to ankylosis of the spine. Historically, AxSpA patients with visible radiographic damage of the sacro-iliac joints on X-ray were classified as AS, whereas patients without this radiologic feature are classified as nonradiographic AxSpA. pSpA is mainly characterized by arthritis of peripheral joints, dactylitis, and enthesitis; in case of PsA, this is associated with skin psoriasis (PSO). Whereas these classifications are very useful in clinical research, it should be noted that in clinical practice the population is very heterogeneous and many patients have a mix of axial and peripheral clinical symptoms, either at presentation or during the evolution of the disease.

CLASSICAL TREATMENT PARADIGM IN SPONDYLOARTHRITIS

The current treatment paradigms in AxSpA and PsA have been extensively reviewed elsewhere [4▪,5▪]. Briefly, nondrug interventions such as physiotherapy and nonsteroidal anti-inflammatory drugs (NSAIDs) are the first-line treatment for both AxSpA and pSpA. Conventional disease-modifying antirheumatic drugs (csDMARDs) are recommended as a next step for patients with peripheral disease, albeit these drugs have mainly been studied in PsA (not other pSpAs) and, even in PsA, the evidence supporting their efficacy is limited to even debatable

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Curr Opin Rheumatol 2018, 30:79–86
DOI:10.1097/BOR.0000000000000457

KEY POINTS

- New biological agents and small molecule inhibitors offer further therapeutic opportunities for the treatment of spondyloarthropathies.
- The availability of new treatment options raises the importance of defining treatment strategies.
- Key aspects to focus on to optimize treatment for spondyloarthropathies include timing and sequence of management, stratified medicine approaches, and treat-to-target strategies.

[6,7]. Local corticosteroids are a useful addition for pSpA. In contrast to pSpA, there is no evidence supporting the use of csDMARDs or corticosteroids in axial disease.

SpA patients with persistent moderate to high disease activity despite the previous treatments are eligible for TNF blocking biologics [Tumor Necrosis Factor inhibitors (TNFi)]. There is ample clinical trial and real world evidence that TNFi have a major clinical impact on both pSpA and AxSpA disease as well as on function and quality of life (QoL). Moreover, TNFi can also have a therapeutic effect on associated symptoms, such as skin PSO in the case of PsA. All five originators of TNFi (infliximab, etanercept, adalimumab, certolizumab pegol, and golimumab) have been approved for treatment of PsA and AS, with the four latter ones also being approved for nonradiographic AxSpA in Europe.

Despite the profound clinical efficacy of TNFi in SpA, a significant proportion of patients have either no response, a partial response with residual disease activity, or a loss of response overtime. Moreover, the use of TNFi may be limited by adverse events, tolerability issues, and/or comorbidities (such as current or recent history of malignancy). This has triggered extensive research for other therapeutic targets, resulting in the recent emergence of new treatment modalities for SpA.

NEW TARGETED THERAPIES IN PSORIATIC ARTHRITIS

In Table 1, we summarize the published phase II/III studies in PsA and AS from recent years. Ustekinumab, a mAb toward the p40 subunit of interleukin (IL)-23 and IL-12, was the first approved non-TNFi biologic in PsA. Two pivotal phase III studies demonstrated the efficacy of ustekinumab on peripheral arthritis, enthesitis, dactylitis, and skin disease in PsA [8,9▪]. Efficacy was maintained overtime, inhibited radiographic progression, and was present albeit lower in patients previously exposed to TNFi. No specific safety signals emerged. In the absence of head-to-head trials with TNFi in PsA, the trend toward lower and slower response with ustekinumab on the joint but its good efficacy on skin may favor the use of this drug in TNFi-incomplete responders (IRs) and in PsA patients with extensive skin disease.

Apremilast, a small molecule drug, is also approved for the treatment of PsA. Apremilast is an inhibitor of phosphodiesterase 4, which allows to modulate a number of key cytokine axes in different immune and effector cells. Again in the

Table 1. Published efficacy data on new compounds in spondyloarthritis

Drug	Target	Disease subtype	Approved	Highest phase published	Study name	Primary endpoint met
Ustekinumab	anti-p14 (IL-23)	PsA	Yes	III	PSUMMIT I/II (Kavanaugh *et al.*, 2016; McInnes *et al.*, 2013)	Yes
		AS	No	II	TOPAS (Poddubnyy *et al.*, 2014) (Open-label proof of concept)	Yes
Apremilast	PDE-4 inhibitor	PsA	Yes	III	PALACE I/II/III (Kavanaugh *et al.*, 2014; Schett *et al.*, 2012)	Yes
		AS	No	II	START (Pathan *et al.*, 2013)	No
Secukinumab	anti-IL-17A	PsA	Yes	III	FUTURE I/II (Mease *et al.*, 2015, Mcinnes, 2015)	Yes
		AS/nrAxSpA	Yes	III	Measure I /II (Baeten *et al.*, 2015)	Yes
Ixekizumab	anti-IL-17A	PsA	No	III	SPIRIT-P 1 and 2 (Mease *et al.*, 2016; Nash *et al.*, 2017)	Yes
		AS	No	N/A		N/A
Abatacept	CTLA-4	PsA	yes	III	ASTRAEA (Mease *et al.*, 2017)	Yes
		AS	No	N/A		N/A

AS, ankylosing spondylitis; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; IL, interleukin; NrAxSpA, nonradiographical axial spondyloarthritis; PDE, phosphodiesterase; PSA, psoriatic arthritis; TOPAS, Toronto Psoriatic Arthritis Screen.

absence of head-to-head trials, clinical efficacy on joints and skin in PsA seems modest in comparison with TNFi [10,11]. No significant effect on dactylitis and enthesitis was seen, and the impact on structural progression has not been evaluated. The oral administration and the lack of monitoring requirements, however, could justify its use as a prebiologic and/or as a treatment for milder disease.

Two mAbs targeting IL-17A have been approved for PSO and have also been tested in PsA. Secukinumab demonstrated good efficacy on arthritis, enthesitis, dactylitis, skin, and QoL in two pivotal trials, and inhibited structural progression in TNFi naïve and TNF-IR [12,13]. This drug has been approved but, as the overall clinical response seems to be similar as TNFi, the exact positioning in the treatment paradigm remains to be further refined. Ixekizumab, another anti-IL-17A, showed very similar clinical and radiographic efficacy; interestingly, this trial included an adalimumab control arm, confirming that at par with TNFi [14]. Ixekizumab is not yet approved for PsA. Both IL-17A blockers are associated with dose-dependent mild fungal infections, which reflects the mechanism of this class of drugs.

Most recently, the cytotoxic T-lymphocyte-associated protein-4-immunoglobulin molecule, abatacept, has been approved for PsA. Abatacept demonstrated clinical efficacy on arthritis, but a smaller benefit on other musculoskeletal symptoms as well as radiographic progression in PsA patients [15▪]. Again, important to note is the only modest effect in comparison with TNFi.

Finally, a number of new drugs are currently in clinical development for PsA. This includes the anti-IL-17RA antibody brodalumab (which reported phase II data but the clinical program was interrupted because of a signal for suicidal ideation) [16], the monoclonal anti-IL17A/F antibody bimekizumab [17], several antibodies toward the p19 (subunit of IL-23) (guselkumab, tildrakizumab, and risankizumab) [18], and several Janus Kinase inhibitor (JAKi) (tofacitinib and baracitinib). If and when these treatments will be approved and will become available in clinical practice, remains to be determined.

NEW TARGETED THERAPIES IN AXIAL SPONDYLOARTHRITIS

The number of emerging treatment options in AxSpA is smaller than in PsA. The only targeted therapy, other than TNFi which has been approved for AS is secukinumab. Two trials showed efficacy on clinical signs and symptoms as well as function and QoL, both in TNFi-naïve and TNFi-IR patient [19,20▪▪]. The safety profile is similar as in PsA, with a signal for mild fungal infections. Further data on radiographic progression [21], head-to-head trials with TNFi, and data in nonradiographic AxSpA would help to define the exact position of this drug in the treatment of AxSpA.

No other drugs have been approved or reported phase III data. Ustekinumab showed preliminary efficacy in an open-label proof of concept study, but phase III data have not yet been released [22]. Apremilast failed to reach its primary endpoint in phase II [23] and phase III data have not yet been released. Ixekizumab is in phase III in AS and nonradiographical AxSpA. As to phase II, tofacitinib showed moderate efficacy but does not seem to progress to phase III [24▪]. Several other compounds, including risankizumab and bimekizumab, are in phase II in AS, without any efficacy data being public at this time.

TREATMENT STRATEGIES IN SPONDYLOARTHRITIS

The emergence of new treatment options in PsA and, to a lesser degree, in AxSpA, opens new avenues and opportunities for treating patients with nonresponse, contraindications, side-effects, or intolerance to classical drugs. However, it raised a couple of additional questions related to the optimal use these drugs. In line with the evolutions in the field of rheumatoid arthritis (RA) treatment over the last decade, it becomes more relevant than ever to define not only drugs and treatment options but also treatment strategies. In particular, are treatment strategies such as early intervention, combination treatment, personalized medicine, treat-to-target, and tight control relevant and useful in SpA? What type of evidence needs to be generated to validate these strategies? And what are the hurdles that may hamper the implementation of these strategies in clinical practice?

EARLY AGGRESSIVE TREATMENT IN SPONDYLOARTHRITIS

In RA, early aggressive treatment did not only result in prevention of structural damage but also in increased clinical response rates, especially with regard to low disease activity and remission [25]. Both aspects are important to achieve a more favorable long-term outcome. A similar concept has not yet fully been established in SpA, partially because early diagnosis remains often a challenge and because NSAID and/or csDMARD are still considered the cornerstones of a gradual step-up treatment paradigm.

The modest and even sometimes debatable efficacy of csDMARD in pSpA triggered a couple of

clinical trials that challenge the concept of gradual step-up therapy. A first study compared the use of infliximab and methotrexate (MTX) with MTX alone in PsA patients naïve for MTX in an open-label fashion [26]. Both arms show a high response rate, although the combination therapy was far superior in achieving remission outcomes. Confirmation of the superior efficacy of TNFi and MTX versus MTX alone awaits confirmation in a double-blind randomized setting.

Based on the demonstration that TNFi are not only effective in PsA but also in other subtypes of pSpA [27–29], the CRESPA study investigated the efficacy of NSAIDs and golimumab versus NSAIDs alone in pSpA patients with very early in the disease [30▪▪]. The study showed a substantially higher remission rate at 24 weeks, with 75% of golimumab-treated patients reaching a status of complete absence of disease symptoms compared with 20% in the NSAID only group ($P < 0.001$).

The concept that early initiation of TNFi may lead to higher remission rates was also explored in AxSpA. Sieper *et al.* [31] showed a superior efficacy of TNFi in early AxSpA versus NSAIDs alone. The INFAST study evaluated the use of infliximab and naproxen with naproxen alone in patients with active early (<3years disease duration) AxSpA. A greater ASAS partial remission response occured in the TNFi group (62 versus 35%) [31]. This study supports the early diagnosis and treatment of SpA with full dose of NSAIDs and a fast escalating combination of NSAID and TNFi treatment in patients with an insufficient response.

Whereas these studies tend to indicate that early aggressive treatment is useful in both pSpA and AxSpA, a couple of key questions remain unanswered. First, are the high remission rates because of the use of more effective drugs (TNFi) and/or to the earlier initiation? Indirect comparison between trials suggest that the remission rate of 35% upon NSAID therapy in the INFAST trial is higher than reported previous with NSAID in established disease (12–15%) [32,33]. Multiple factors may bias this comparison and direct analysis is mandatory to come to firm conclusions. Second, is early aggressive treatment also associated with reduced radiographic progression? Whereas most targeted therapies, with the exception of apremilast, have demonstrated an impact on progression of structural damage in established PsA, this is not the case for new bone formation in AxSpA. Certainly for this subgroup, it would thus be crucial to see if earlier initiation of TNFi may have a significant impact on structural damage overtime. Third, the few early intervention studies described above were all conducted with TNFi, leaving the question open whether the same concept holds true for other MoAs. Fourth, it needs to be better determined if 'immediate' initiation of targeted therapies is required to obtain high remission rates in SpA, or if it would be sufficient to decrease the time intervals in the current step-up approach? For example, the INFAST study showed that response to NSAIDs is noticed within the first 2–4weeks and the need for a TNFi could be considered in an early state [31]. Similarly, should csDMARDS really be used for 6 months before escalating to a targeted therapy in PsA [34]. Finally, an important and unanswered question is if rapid induction of remission upon early aggressive treatment may allow tapering and/or stopping of the targeted therapy overtime? The targeted therapy would then be used in an 'induction' strategy, with maintenance using NSAIDs and/or csDMARDs.

Beyond these scientific questions about early aggressive treatment strategies in SpA, one should also consider potential barriers to implement this in clinical practice. Early diagnosis, which is still a major challenge in AxSpA [35▪], early referral of PSO patients with musculoskeletal symptoms and access to targeted therapies are just a few examples of obvious challenges that need to be addressed to use this strategy effectively [36–38].

SEQUENTIAL VERSUS COMBINATION THERAPY IN SPONDYLOARTHRITIS

Another ongoing discussion is whether the use of a concomitant csDMARD might increase response rates or prolong drug survival of biologics in SpA. In RA, the continuation of TNFi with MTX is supported by current treatment guidelines because the combination is proven to be more effective than anti-TNF monotherapy. Two prospective cohorts in PsA reported that patients already taking not using concomitant MTX before starting TNFi was associated with a poorer clinical response [39,40]. However, other studies could not confirm this [41], emphasizing the inherent biases and limitations of such cohort studies and the importance of prospective randomized trials. In PsA, multiple randomized controlled trials (RCTs) showed no difference in response rates to TNFi or other biologics in patients with and without concomitant use of MTX [42–44]. Similar results were reported in an AS trial with infliximab [45]. The key limitations, however, is that they included patients who failed already on MTX. We do not have a randomized prospective study comparing a targeted therapy versus a targeted therapy with concomitant MTX. We also lack prospective randomized data on tapering/stopping MTX in patients with good clinical response to a biologic.

Considering the fact that MTX is not effective for AxSpA, the benefit/risk balance of MTX combination versus mono/sequential therapy is more relevant to PsA than to AxSpA. However, similar questions can be raised about the combination of NSAIDs and TNFi in AxSpA: Should TNFi be added to NSAIDs or replace it in IRs? And could NSAIDs be stopped when a patients is in remission with the combination therapy?

Adding further complexity to the treatment strategy, we have no evidence that the potential benefit of combination of MTX and/or NSAIDs with a TNFi in SpA would also translate to a similar benefit with other biologics such as IL-17i or ustekinumab. Moreover, we also lack data on the combination of other synthetic drugs, such as leflunomide but certainly also newer compounds such as apremilast or JAKi, with TNFi and IL-17i. Finally, combination of biologics failed to increase efficacy but jeopardized safety in couple of RA trials [46–48], but may be a realistic option in SpA considering the difference in disease, comorbidities, and MoAs. Case reports suggest, for example, that combination of ustekinumab with TNFi might be considered in refractory PsA [49]. And bispecific antibodies blocking TNF and IL-17A, and IL-17A and IL-17F are in clinical trials in PsA and AxSpA.

In terms of potential hurdles for implementation of combination treatment strategy, one should consider access, costs, tolerability, and safety. It should also be better defined in which patients such combination treatments hit the right benefit/risk/cost balance and, in particular, whether all patients or only refractory patients would be eligible for such an approach.

PERSONALIZED/STRATIFIED MEDICINE IN SPONDYLOARTHRITIS

This raises another important treatment strategy question: should distinct subpopulations of SpA patients require different treatments? The global answer to this question is definitively yes as it is evident that not all treatments are as effective for peripheral versus axial disease, with as prototypical example csDMARDs. The question, however, is if and how these strata could be further refined.

Within axial disease there is no evidence that AS and nonradiographic axSpA respond differently to specific treatments. This has been best evidenced for TNFi [50]. There are, currently, no data on IL-17i in nonradiographical AxSpA but it is reasonable to expect a similar efficacy as in AS. The same holds through for pSpA: different TNFi have demonstrated efficacy not only in PsA but also other pSpA [27,28,30▪▪,51,52]. Whether this also applies to other drugs approved for PsA, remains to be determined. An additional question in pSpA is if patients should be stratified according to arthritis versus enthesitis/dactylitis. Based on animal data, it has been hypothesized that enthesitis may be more IL-23/IL-17 dependent [53]. However, other models indicated both the relevance of IL-23 for synovitis and the relevance of TNF for enthesitis [54]. Accordingly, significant improvements in enthesitis/dactylitis have been seen in PsA trials with both TNFi [55,56], ustekinumab [57], and IL-17i [9▪,14]. In the absence of clear head-to-head data, there is thus no strong evidence to for such treatment stratification.

Beyond axial and peripheral disease, the presence of extraarticular manifestations represent a clear base for a stratified treatment strategy in SpA. Skin PSO can be treated with both TNFi and drugs targeted the IL-23/IL-17 axis, but the latter have shown superiority in head-to-head trials in PsO [58,59] and may thus favor their use in PsA patients with extensive and/or refractory skin disease. In contrast, apremilast and abatacept have only limited efficacy on skin [10,15▪]. For SpA-associated gut inflammation, monoclonal anti-TNF antibodies and ustekinumab have shown clear efficacy in Crohn's disease [60,61], which is not the case for etanercept and secukinumab [62,63]. Although less strongly supported by direct evidence from RCTs, monoclonal anti-TNF antibodies may also be more effective for the treatment of uveitis [64–67].

An additional dimension for stratification is the presence and/or prognosis of structural damage. In PsA, most targeted therapies have demonstrated impact on structural progression with the exception of apremilast, questioning the use of the latter drug in patients with erosions and/or poor prognostic factors. In AxSpA, the question may become even more important as, despite some debate, TNFi have no proven impact on osteoproliferation [68,69], whereas preclinical data [70] and very preliminary clinical evidence [20▪▪] suggest that IL-17i such as secukinumab may have a more profound impact. This hypothesis requires now mandatory confirmation in well designed clinical trials. If confirmed, it could help to position the use of TNFi versus IL-17i in AxSpA without or with signs of rapid structural progression, respectively.

A final and clinically very relevant form of stratification is to adapt the treatment strategy to previous treatments, in particular for TNFi. Previously, the only option in PsA or AxSpA patient with incomplete response to a first TNFi was to switch to another TNFi. Although guidelines do recommend switching strategies in TNFi nonresponders, there is a lack of prospective controlled data to support

these [71,72]. Randomized studies with both secukinumab and ustekinumab in AS and PsA have demonstrated significant responses in TNF-naive and TNFi-IR patients. Whereas these data prove that these treatments are good options for TNFi-IR, further studies need to compare switch to a second TNFi versus to another MoA to determine the best treatment strategy.

As to implementation of a stratified/personalized treatment strategy in clinical practice, obvious hurdles are the fact that the phenotype of the disease may be mixed and may even vary overtime in a single individual. More importantly, the biggest obstacle may be the fact that volume rather than value-based contract by payers and insurance companies may not allow healthcare professionals to use all available treatment options in a personalized medicine approach.

TREAT-TO-TARGET AND TIGHT CONTROL IN SPONDYLOARTHRITIS

Treat-to-target is a very successful treatment strategy in the treatment of RA. Treat-to-target is treatment strategy, where the clinician treats the disease aggressively enough to reach and maintain a prespecified and sequentially monitored target. In RA, treat-to-target improves clinical outcomes and limits radiographic progression [73]. Treatment recommendations recommend to treat-to-target in SpA although evidence supporting the beneficial effect over standard care is limited in this patient group [74▪,75▪▪]. The tight control of PsA trial is the first treat-to-target study in SpA and demonstrated that the tight control of disease activity of PsA through a treat-to-target approach significantly improves clinical outcomes for patients with early disease in comparison with standard care [76▪▪]. However, an effect on enthesitis/dactylitis or radiographical outcome was not different between the groups. And the treat-to-target approach increased the occurrence of adverse events. Further studies are needed to confirm the clinical and long-term benefits of treat-to-target in the several SpA subtypes as well as the cost, risk, and additional adverse event burden of this approach.

Moreover, several potential hurdles to implement the treat-to-target strategy in clinical practice remain. For example, consensus on the definition of remission and response criteria to use is still in debate [77▪]. One should consider costs (increase in agents used and more outpatient visits), outweigh risk of increase in adverse events versus clinical benefit, feasibility in clinical practice and the willingness of rheumatologist to implement this strategy, and the availability of the treatments.

CONCLUSION

The rapid expansion of the therapeutic options in SpA carries a lot of promise for our patients with peripheral and/or axial disease. At the same time, this expansion emphasizes the fact that not only the treatment as such but also the treatment strategy is crucial to reveal the full therapeutic potential and benefit for patients. Key aspects to develop optimal treatment strategies in SpA relate to timing and sequence of treatment (including combination treatments), stratified medicine approaches, and treat-to-target strategies. Whereas cautious but crucial steps have been taken in the last years to explore these aspects, it is now time for full-scale investment in prospective strategy trials. Finally, it will be critical to connect the strategy trial outcomes with real world evidence (cohort studies, payer and access ecosystem), to identify and overcome issues that may complicate or even prevent the implementation of these treatment strategies in clinical practice.

Acknowledgements

None.

Financial support and sponsorship

M.vd.S. has received speakers/consultancy fees from Takeda, Tillotts, Benecke MSD. D.L.P.B. has received research grants and speakers/consultancy fees from Pfizer, MSD, AbbVie, UCB, Novartis, Janssen, Boehringer Ingelheim, speakers/consultancy fees from Eli Lilly, Roche, BMS, Glenmark, and is currently and employee of UCB.

Conflicts of interest

There are no conflicts of interest.

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- ▪ of special interest
- ▪▪ of outstanding interest

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Psoriatic arthritis: new evidence for old concepts

Enrique R. Soriano, Josefina Marin, and Maria L. Acosta-Felquer

Purpose of review
The review gives an updated overview of some of the new concepts in the management of psoriatic arthritis (PsA): early diagnosis, remission as an objective, treat-to-target, and treatment guidelines.

Recent findings
Early diagnosis, targeting remission as part of a treatment strategy, and new guidelines providing evidence-based support to these concepts are main topics in recent publications.

Summary
Dermatologists and rheumatologists should work together to reduce the number of patients remaining undiagnosed, and the time to do so.
Remission definition in PsA is still controversial. There is good evidence and convincing arguments for both multidimensional measures, such as minimal disease activity, or unidimensional ones, as disease activity index for PsA. New data on the analysis of tight control of inflammation in early PsA trial showed that the strategy might not be cost-effective on the short term, and that oligoarthritis is less benefited.
The new European League Against Rheumatism and Group for Research and Assessment of Psoriasis and PsA recommendations exhibit differences. Methotrexate and tumor necrosis factor inhibitors are favored in European League Against Rheumatism guidelines, whereas other conventional synthetic disease-modifying antirheumatic drugs and biologics are equally positioned in Group for Research and Assessment of Psoriasis and PsA recommendations.

Keywords
psoriatic arthritis, recommendations, remission, treat-to-target

INTRODUCTION

Psoriatic arthritis (PsA) is a complex and heterogeneous disease that involves several different domains, including skin, nails, enthesis, spine, and peripheral joints, among many others.

During the last years more research has focused in supporting concepts for PsA, that have already been well accepted and embraced by the rheumatologists in rheumatoid arthritis; including: early diagnosis, remission as a treatment objective, and the 'treat-to-target' concept.

Definition of remission is intimately linked to treat-to-target strategy, as treatment should be adjusted to achieve that definition. Unfortunately, there is still not universal agreement on how remission should be defined in PsA.

Very recently an international task force published the updated recommendations of treating axial and peripheral spondyloarthritis, especially PsA, to target [1▪]. Despite, that there was some improvement compared to 2012, for most of the recommendations the level of evidence was still very low. This indicates that much more research is needed to support these logical principles.

New guidelines have been recently published, updating old ones. Despite striking similarities some important divergences were identified, showing that even looking at the same evidence, different recommendations could arise from different groups.

In this review, we discuss the most recent evidence supporting each one of these concepts.

EARLY DIAGNOSIS

PsA is associated with progressive joint damage, reduced quality of life and function, and increased mortality [2]. Several observational studies have shown that early diagnosis improves long-term outcomes [3]. Haroon *et al.* [4] demonstrated that even a 6-month delay in rheumatologic consultation was

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Curr Opin Rheumatol 2018, 30:87–93
DOI:10.1097/BOR.0000000000000468

KEY POINTS

- For early diagnosis, every rheumatologists and dermatologists must find the best way to work together.
- As no universal definition of remission is accepted, each rheumatologist should choose a validated one as their targeted objective.
- A treat-to-target strategy is strongly recommended, although it might not be cost-effective in the short term.
- New GRAPPA and EULAR guidelines, although with some differences, provide strong evidence-based support for the current treatment of PsA.

associated with more disability, more erosions, and more sacroiliitis.

In the vast majority of PsA patients, psoriatic cutaneous lesions precede development of arthritis. However, around 30% of psoriasis (PsO) patients seen regularly, even in experienced dermatology centers, have undiagnosed PsA [5,6]. To try to solve this problem, numerous screening tools have been developed to identify PsA in patients with PsO [7–10] and in the general population [11]. However, although these screening tools were very sensitive and specific in their development programs, when were compared in independent settings, they did not function very well [3,12]. Perhaps a way to go is to train dermatologists to assess the presence of PsA with a few questions and clinical examination [13], and to prepare rheumatologists to receive patients with PsO with rheumatic conditions other than PsA. At the end, osteoarthritis and fibromyalgia are managed by rheumatologists in most parts of the world.

Ultrasound (US) has been extensively studied in patients with PsO and PsA, as shown in a recent systematic review by the Italian Society of Rhematology [14▪]. Subclinical lesions (synovitis, erosions, and enthesopathy) in special enthesopathy are frequently found in patients with PsO, although little is known related to the prognostic value of these features [14▪]. Only one longitudinal study investigated the predictive value of US in diagnosing PsA in patients with PsO [15]. After 2 years of follow-up, about 23% of patients (seven of 28 patients) with PsO developed PsA according the ClASsification of Psoriatic ARthritis criteria, baseline thickness of the quadriceps tendon was found to be an independent predictor of the development of PsA [15].

A close anatomical relationship between nail involvement and enthesopathy at the distal interphalangeal joints level had been extensively described, supporting the synovio-enthesial concept [16–19].

Despite all these studies, there is still no clear indication for the use of US in PsA screening in completely asymptomatic PsO patients, as the risk of developing PsA in those patients with abnormal features is still not clear. However, when a patient with PsO refers musculoskeletal symptoms (arthralgias/arthritis, tendinitis, and so on) the use of US clearly helps to define the lesion, and allows the early diagnosis of PsA. It is our view that a patient with PsO and arthralgias, with US synovitis, should be considered as a PsA patient. In the future, when the predictive value of each one of the US features would be established, we believe that the use of US as a screening tool would be justified and rapidly adopted.

Another key point in early diagnosis is patients' awareness of PsO, PsA, and their relationship. Patient advocacy organizations play a vital role in disease awareness [20]. (Fig. 1).

TREAT-TO-TARGET

The principle of treat-to-target has been successfully applied to some of the most common diseases, including arterial hypertension, coronary artery disease, diabetes, and rheumatoid arthritis. Identifying appropriate therapeutic targets and pursuing these aggressively have led to superior clinical outcomes for patients with these diseases.

One of the key issue in PsA is identifying the appropriate therapeutic target. To be appropriate a target should fulfill some characteristics: should be a validated and objective measurement, surrogate of

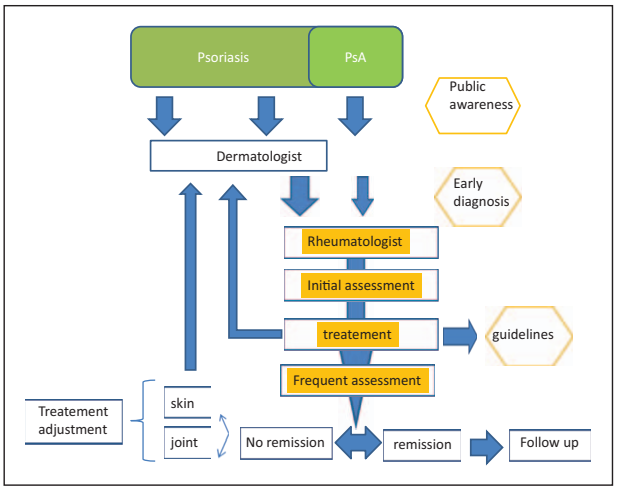

FIGURE 1. Flow chart, including rheumatologists–dermatologists interaction, early diagnosis, treat-to-target concepts, and guidelines. The chart describes an ideal relationship between all these components. PsA, psoriatic arthritis.

Table 1. Components of some of the assessment tools proposed for psoriatic arthritis

	Arthritis (swollen/tender joint counts)	Skin disease (PASI/BSA)	Enthesitis	Patient's global disease activity assessment (VAS)	Patient's pain assessment (VAS)	Physical Function (HAQ)	Physician's global disease activity assessment (VAS)	Acute-phase response	Dactylitis	Spinal disease
MDA	66/68	√	√	√	√	√	X	X	X	X
DAPSA	66/68	X	X	√	√	X	X	√	X	X
CPDAI	66/68	√	√	X	X	√	X	X	√	√
DAS28	28	X	X	√	X	X	X	√	X	X
PASDAS	66/68	X	√	√	X	X	√	√	√	X

BSA, body surface area; CPDAI, composite psoriatic disease activity index; DAPSA, disease activity index for psoriatic arthritis; DAS28, disease activity score 28 joints; HAQ, health assessment questionnaire disability index; MDA, minimal disease activity; PASDAS, psoriatic arthritis disease activity score; PASI, psoriasis area severity index; VAS, visual analogue scale.

the outcome desired for that disease; and should be feasible to measure in daily clinical practice, as it would need to be assessed frequently.

Both recently published guidelines: Group for Research and Assessment of Psoriasis and PsA (GRAPPA) and European League Against Rheumatism (EULAR), incorporated the concept of treat-to-target in their overarching principles, or recommendations [21,22]. However, none of them defined which target should be used. The updated recommendations on treat-to-target in spondyloarthritis [1▪] suggested the use of minimal disease activity (MDA) [23], or disease activity index for PsA (DAPSA) [24], (Table 1) as the targets for the application of treat-to-target in PsA, although accepted only by a simple majority of 51.6% of the task force participants [1▪]. Do these two measures meet the criteria for a good target? MDA was validated using interventional trial data from the infliximab and golimumab trials [25,26▪]. Irrespective of treatment randomization, achievement of MDA was associated with significantly less radiographic progression in both studies. Reduced joint damage in those patients achieving sustained MDA was also demonstrated in an observational cohort study [27]. In a cross-sectional multicenter Spanish study, achievement of MDA was associated with lower impact of the disease as assessed by the PsA impact of disease questionnaire [28]. Although there is no data on feasibility observational studies on real-world data have shown that MDA is feasible to use in daily clinical practice [29,30]. DAPSA was initially validated in an infliximab trial, where it showed good correlation with other disease activity measures and sensitivity to change [24]. More recently DAPSA was validated against functional status and radiographic progression in the IMPACT II (infliximab in PsA trial) and golimumab trials, showing that higher DAPSA scores were significantly and independently associated with probability of structural progression [31▪]. Again, as in MDA, there is evidence from real-world data that DAPSA is feasible in clinical practice [29,30,32]. We could conclude that both proposed tools fulfill criteria for a good target. There are some differences among the two measurements. MDA includes in an explicit way assessment of some domains that are not included in DAPSA score.

Whether to include in a composite index all (or many) PsA domains is a matter of debate among experts [1▪,33,34]. Although some experts favor unidimensional composite measures, others (ourselves among them) think that a multidimensional measure such as composite psoriatic disease activity index, PsA disease activity score, or MDA allows physicians to see individual domain responses while providing a comprehensive measure of

inflammatory disease activity. One of the strongest arguments favoring multidimensional measures is that recommendations are also important to educate rheumatologists, and many of them would only assess what is suggested by the recommendation, so if the skin or enthesis are left out of the definition of remission, they would probably not be evaluated.

The evidence for treat-to-target in PsA comes from the tight control of inflammation in early PsA (TICOPA) trial [35]. Patients with early disease-modifying antirheumatic drug-naïve PsA)were randomized 1 : 1 to either tight control (4-weekly review with therapy escalation if MDA criteria not met) or standard care (12-weekly review). Patients in the tight control arm showed a significant benefit in peripheral joint disease activity, skin disease activity, and greater improvements in quality of life and patient-reported functional ability [35,36]. There was no difference on radiographic progression between arms, but at baseline patients had minimal radiographic damage, and little progression over 48-weeks [36]. A higher proportion of patients in the tight control group than in the standard care group were treated with combination of disease-modifying antirheumatic drugs and biological therapies [35]. Adverse events were reported more often by patients in the tight control group than in the standard care group (97 vs. 77%) [35]. Also among those patients with an adverse event a higher median number of adverse events was reported in the tight control arm (six vs. three events). The most commonly reported adverse events were nausea, liver abnormalities, and infections [35].

Two recent papers published from new analysis of the TICOPA trial call for some caution on the enthusiasm with treat-to-target in PsA. O'Dawyer *et al*. [37] published the cost-effectiveness analysis of TICOPA trial. The study did not find a tight control strategy in PsA to be cost-effective neither in the basic, nor in most of the sensitivity analysis. The higher use of high-cost tumor necrosis factor (TNF)-α inhibitors, and the little difference in Quality Adjusted Life Years gained between both arms, appeared to be key factors in the negative result. Only significative reductions in biologic acquisition costs, and diminishing the number of visits (something that might be against the tight control itself) seemed to produce cost-effective figures [37]. However, results were analyzed only at 48 weeks, and no modeling over long term was performed. Being MDA, an intermedia surrogate outcome associated with preservation of quality of life and no radiographic progression, long term analysis is expected to be cost-effective.

In the other study, dynamics of response of different composite measures were analyzed, specially in patients with oligoarthritis [38]. Although significant difference between groups was seen for each measure, the significance levels were greatly diminished in patients with oligoarthritis. The composite scores were lower at baseline, as would be expected, but the early difference seen for the entire cohort was not seen for the oligoarthritis subgroup alone. This subanalysis raises the question of whether this strategy applies equally to this common subgroup of patients. More research is needed in patients with oligoarthritis.

NEW GUIDELINES

Recently the new GRAPPA and EULAR recommendations on PsA treatment were published [21,22]. Although both guidelines are evidence based and have many similarities, some differences deserve consideration [39▪] (Table 2). This divergence is not surprising, as although clinical trials provide data on the efficacy and safety of treatments, the production of final recommendations is a process that involves balanced interpretation, careful analysis, discussion, and consensus [39▪].

The first difference is related to the composition of both task groups. GRAPPA is an international organization that includes dermatologists and rheumatologists from all around the world, whereas EULAR is a rheumatologists' organization from Europe. GRAPPA task force included a respectable number of dermatologists and rheumatologists from distinct parts of the world, whereas EULAR's one only included one dermatologists and European rheumatologists. Therefore, EULAR recommendations were focused on musculoskeletal manifestations and GRAPPA one's included skin and nail involvement. Both task forces included patients [39▪].

Overarching principles were included in both guidelines and were similar including heterogeneity of PsA, collaboration among practitioners in management, and shared decision-making with patients; however, GRAPPA principles more clearly stressed the importance of assessment of all disease domains [21].

GRAPPA recommendations moved away from stratification by disease severity among different domains developed in the previous guideline [40], as it was considered that there is not enough evidence for clear cutoffs to separate different severity groups, and for different recommendations for each one of them [21]. Some prognostic factors remain, modifying treatment choices. In the EULAR recommendations, the algorithm considers disease severity and poor prognostic factors (defined as five or more actively involved joints that are tender or

Table 2. Comparison of Group for Research and Assessment of Psoriasis and psoriatic arthritis and European League Against Rheumatism simplified treatment algorithms

Clinical Involvement	GRAPPA	EULAR
Predominant peripheral arthritis		
DMARDs naive	csDMARDs (MTX, LFN, SSZ)	CsDMARDs (MTX: preferred)
	Poor prognostic factors: TNFi	
DMARDs failure	TNFi; IL 12–23i; IL17i; PDE4i	Adverse prognostic factors: TNFi preferred; IL 12–23i; IL17i(bDMARDs): if TNFi contraindicated PDE4i if bDMARDs contraindicated
		No adverse prognostic factors:
bDMARDs failure	Switch bDMARDs	Switch bDMARDs or PDE4i
Predominant axial		
NSAIDs naïve	NSAIDs	NSAIDs
NSAIDs failure	TNFi; IL 12–23i; IL17i	TNFi preferred; IL 12–23i; IL17i: if TNFi contraindicated
bDMARDSs failure	Switch bDMARDs	Switch bDMARDs
Predominant enthesal		
NSAIDs naïve	NSAIDs	NSAIDs
NSAIDs failure	TNFi; IL 12–23i; IL17i; PD4i	TNFi preferred; IL 12–23i; IL17i: if TNFi contraindicated PDE4i if bDMARDs contraindicated
bDMARDs failure	Switch bDMARDs or PDE4i	Switch bDMARDs or PDE4i

bDMARD, biologic DMARD; csDMARD, conventional synthetic DMARD; DMARD, disease-modifying antirheumatic drug; EULAR, European League Against Rheumatism; GRAPPA, Group for Research and Assessment of Psoriasis and psoriatic arthritis; IL 12–23i, interleukin 12–23 inhibitor; IL17i, interleukin 17 inhibitor; LFN, leflunomide; MTX, methotrexate; PDE4i, phosphodiesterase 4 inhibitor; SSZ, sulfasalazine; TNFi, tumor necrosis factor inhibitor.
Adapted with permission [21] and [22].

swollen; radiographic damage, elevated levels of acute-phase reactants, and extraarticular manifestations of PsA, particularly dactylitis) [22].

Although EULAR guidelines mainly focus on peripheral arthritis, with a single flow chart, that marginally includes enthesitis and axial disease, GRAPPA recommendations provide six separate flow charts, one for each treatment domain (peripheral arthritis, axial disease, enthesitis, dactylitis, and skin and nail involvement) presented side by side, with the aim that therapies should target as many active domains as possible [39▪].

Both guidelines recommended a step-up approach. For peripheral arthritis, conventional synthetic disease-modifying antirheumatic drug (csDMARDs) are recommended as first-line therapy. [21,39▪,41]. Although EULAR recommended methotrexate as the preferred csDMARD, based mainly on observational data, GRAPPA committee felt that this drug could not be considered superior to other csDMARDs on the basis of the available evidence, and suggested methotrexate, leflunomide, or sulfasalazine with the same strength of recommendation [21,39▪]. In GRAPPA treatment algorithm, patients with severe or poor prognosis can be prescribed biologic agents as first-line therapy without having been given a csDMARD. The EULAR group does not allow prescribing a biologic before a csDMARD for peripheral disease, as there is no high-level evidence showing major progression of damage or disability, from delaying therapy with biologic agents for a few months, by prescribing methotrexate first [21,22,39▪]. Although a biologic agent, a targeted synthetic DMARDs (tsDMARDs), or both are suggested by both guidelines in those whom csDMARDs are insufficient, some differences are evident. TNF inhibitors are given preference as first-line biologic therapy in the EULAR recommendations, based on the longer duration of experience with these drugs and the greater quantity of long-term efficacy and safety data available in comparison with newer biologic agents [22]. In the GRAPPA recommendations, TNF inhibitors and other biologics are included in the same 'step' in the GRAPPA flow charts, which enables newer biologic agents to be used as first-line therapy over TNF inhibitors [21,22,39▪]. Longer duration of experience is difficult to quantify, not all TNF inhibitors have been on the market for the same amount of time or have the same quantity of long-term efficacy or safety data, and when or how to make the cutoff point in time, to move from one situation to the other is not determined.

There are also some differences on the recommendation on the use of apremilast, an oral tsDMARD that inhibits phosphodiesterase 4 [39▪]. Although EULAR recommended that this drug should only be prescribed to patients who do not achieve treatment targets with csDMARDs, and for whom biologic agents are not appropriate [22], apremilast received a 'strong' recommendation by GRAPPA for patients with peripheral arthritis unresponsive to csDMARDs, and a 'conditional' recommendation for patients with peripheral arthritis who were DMARD naïve [21,39▪]. For these decisions, whereas GRAPPA focused on ease of use and safety, EULAR placed emphasis on efficacy, lack of radiographic data, and cost–benefit ratio [39▪].

For enthesitis and axial involvement both guidelines agree that there is no evidence to use csDMARDs as first-line therapy and that those patients should receive biologic agents, tsDMARDs, or both [21,22,39▪]. Again, in a similar way to what happened in peripheral joint involvement, there were differences in the proposed order, EULAR favoring TNF inhibitors first, and GRAPPA positioning all of them at the same level [21,22,39▪].

CONCLUSION

For early diagnosis rheumatologist and dermatologist should work together, different models could be found to try to capture the pool of undiagnosed patients with PsA. US is not recommended nowadays for screening PsO for PsA diagnosis.

Although everybody agrees that remission should be the treatment objective in PsA, there is still controversy in how to measure it. However, it is clear that some of the validated measurements should be used, following the treat–to-target strategy.

The new EULAR and GRAPPA recommendations provide rheumatologists with up-to-date evidence-based guidelines, to treat patients with PsA. Although there are some clear differences, reflecting distinct group points of view, in general they are very similar, and clinicians could choose all or parts of any of them with much certainty of doing well. Both guidelines also identified a large research agenda, that needs to be completed in the near future if we want to fulfill many of our knowledge gaps.

Acknowledgements

None.

Financial support and sponsorship

The work was supported by the Rheumatology Unit, Internal Medical Services, Hospital Italiano de Buenos Aires, Argentina.

E.R.S. has received honoraria as speaker, grants or participated in advisory boards, from Abbvie, BMS, Glaxo SK, Janssen, Novartis, Pfizer, Roche, and UCB. J.M. has received honoraria as speaker from Abbvie and Novartis. M.L.A.F. has received honoraria as speaker from Abbvie.

Conflicts of interest

There are no conflicts of interest.

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The complexities of fibromyalgia and its comorbidities

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Purpose of review
Fibromyalgia syndrome (FMS) is defined as chronic, widespread musculoskeletal pain and tenderness with concomitant mood and cognitive dysfunction. Several comorbidities have been reported to be associated with FMS. We reviewed the literature concerning the most noteworthy chronic conditions associated with FMS.

Recent findings
There is mounting evidence displaying the concurrence of fibromyalgia and coexisting medical and psychiatric conditions. Such comorbidities may blur the classical clinical presentations and erroneously lead to misinterpretation of disease activity. The recognition of this fact should be underlined, as misrecognition may lead to excessive therapy and avoidable side-effects of medications on the one hand and to a better handling of FMS on the other hand, leading to improved clinical outcomes.

Summary
A greater proportion of psychiatric and rheumatologic disorders are associated with FMS patients than the population. Consequently, physicians treating patients with either condition should keep in mind that these patients may have such comorbidities and should be treated accordingly.

Keywords
comorbidities, depression, fibromyalgia, musculoskeletal pain, somatoform disorders

INTRODUCTION

Chronic pain is among the most frequent complaints encountered by physicians of all disciplines. The International Association for the Study of Pain (IASP) estimated that chronic pain, including musculoskeletal and joint pain, neck and back pain, cancer pain, trauma and postsurgical pain and chronic headache afflicts approximately 20% (10–55%) of the adult population worldwide [1]. The fibromyalgia syndrome (FMS) is a common chronic pain syndrome and a major contributor to the high prevalence of chronic pain [2].

FMS is characterized by widespread pain, tenderness and diffuse stiffness. Other associated symptoms include mood, cognitive and functional impairments, such as fatigue, sleep disturbances and headaches [1–3,4▪,5–7]. The prevalence rate of FMS in the general population is estimated to be 2–4% [8]. Ablin *et al.* [9] reported a 2.5% prevalence of FMS in the adult population in Israel, with a clear female predominance; nevertheless, FMS is also observed in men. Much research has been conducted in the past decade in order to understand the factors that contribute to the pathogenesis of FMS. Many reports indicate that familial, genetic, environmental, endocrine and neurological factors (both central and autonomic) play a role in the emergence of FMS [10]. Among the neuroendocrine factors thought to be associated with this condition are serotonin deficiency, surplus of substance P, spontaneous nerve activity, expanded receptive fields, *N*-methyl-D-aspartate receptor triggering and decreased growth hormone concentration [2].

Several studies have described in general the comorbidities related to FMS [11,12▪▪,13,14]. In our review, we choose to highlight the psychiatric and organic comorbidities the clinicians should be aware of.

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Curr Opin Rheumatol 2018, 30:94–100
DOI:10.1097/BOR.0000000000000464

KEY POINTS

- Fibromyalgia patients experience higher rate of comorbid conditions, especially rheumatic and psychiatric as compared with the general population.
- Very often, clinicians tend to overlook somatic symptoms in patients and refer them to the fibromyalgia rather than other diseases.
- Given the high comorbidity rates with other illnesses, physicians should keep in mind the associations between fibromyalgia and other somatic disorders and seek for comorbid diseases while treating patients of each disorder.
- It has been shown that treating those comorbidities may also improve fibromyalgia symptoms severity.

Psychiatric comorbidities

Although rates of FMS and psychiatric comorbidities vary among studies, most reported a higher proportion of psychiatric disorders among FMS patients than controls [15–22,23▪,24,25▪].

An Italian group found a correlation between lifetime exposure to traumatic events and post traumatic stress disorder (PTSD) symptoms, as well as to the severity of FMS [26]. A German study included eight medical centres and showed that 45.3% of FMS patients and 3.0% of controls met the criteria for PTSD [11]. This study also looked at the rates of mental disorders and FMS, and found that 65.7% of FMS patients met the criteria for a depressive disorder, 67.9% for an anxiety disorder and 45.5% for PTSD [27].

Researchers from São Paulo studied the association between a major depressive episode and FMS and reported that among FMS patients, 66% were taking antidepressants, 8.6% benzodiazepine and 23.1% antipsychotics. A major depressive episode was diagnosed in 40.5% of FMS patients, while anxiety disorder was detected in 15.9% [28]. Soriano-Maldonado *et al.* [16] examined 451 women with FMS in a cross-sectional study and showed that FMS patients who had various features of comorbid depression also experienced higher pain intensity, fatigue and poor sleep quality compared with their counterparts with minimal signs of depression.

Alciati *et al.* [29] found that 70% of FMS patients were screened positive for various components of the bipolar disorder, especially hypomania, based on a DSM-IV modified interview, suggesting that an overactive lifestyle in FMS patients might actually represent a key component of bipolar disease.

In Turkey, a few studies elaborated on the link between FMS, anxiety and depression. One study showed that FMS patients with rheumatic symptoms had more anxiety, depression and somatic symptoms, as well as higher neuropathic pain scores [30]. A second study recruited 95 FMS patients and 95 controls who completed anxiety, depression and pain questionnaires, as well as the Fibromyalgia Impact questionnaire (FIQ). Health anxiety and depression scores were more than twice higher in FMS patients than in healthy controls. The authors suggested that psychiatric support should be provided to FMS patients, including combined biological, social and psychological care [31].

Rheumatologic comorbidities

The association between FMS and rheumatologic diseases is also well established and has been evaluated extensively in many studies [32,33▪▪,34–39,40▪,41–43]. A Turkish group focusing on FMS and rheumatic diseases investigated 835 patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), ankylosing spondylitis, osteoarthritis, familial Mediterranean fever (FMF), Behçet's disease, gout, Sjögren's syndrome, vasculitis, polymyalgia rheumatica or polymyositis, with or without FMS. FMS prevalence ranged from 1.4 to 25% (lowest for gout, highest for vasculitis). Except for SLE and FMF, disease activity scores of the concomitant rheumatic conditions were significantly higher among all FMS patients than non-FMS patients. Somatic symptoms in rheumatic patients with FMS were very common; they all described abdominal pain, as well as cramps (44.73%), gastric disturbances (42.1%), oral ulcers (26.31%), photosenstivity and Raynaud's syndrome (11.84%) [44]. Another Turkish group found that FMS patients had a higher frequency of rheumatic symptoms, such as photosensitivity, Raynaud's phenomena and ANA positivity when compared with healthy controls, but lower than that found in SLE patients. FMS patients with Raynaud phenomena exhibited higher rates of anxiety, depression, somatic symptoms and neuropathic pain than others did [30].

A cross-sectional study observed concomitant FMS in 23% of patients with rheumatic diseases. Their mean visual analogue scale scores for pain, fatigue and function were almost twice as high as those in patients who had no features of FMS [45].

A prospective observational study on rheumatic patients in Korea used the 1990 and 2010 ACR criteria, as well as clinicians' judgement to establish the diagnosis of FMS. They reported that 30% of patients complained of widespread pain. Among this subgroup of patients with widespread pain, FMS was diagnosed in 34.2% according to the ACR criteria, and in 22.0% based on clinical judgement alone. The occurrence of FMS among rheumatic patients was highest in patients with Sjögren's

syndrome (33.3–66.7%) and with systemic sclerosis (57.1–71.4%) [46].

Another group found that RA patients who screened positive for FMS had higher levels of depression, but similar scores for joint damage. They concluded that in their cohort, FMS traits were mediated by depression rather than by the rheumatoid disease activity [47]. Another study compared the impact of fatigue on the quality of life in FMS and RA patients. They revealed that although fatigue severity was similar in both groups, it had a higher impact on cognitive function in patients with FMS. The researchers concluded that pain and fatigue cause FMS patients to attribute poorer mental health to themselves [48].

In their study, Wach *et al.* [43] reported a high prevalence of 17.5% spondyloarthritis patients with comorbid FMS. This result is in accordance with few previous studies showing prevalence of 4.1% to as high as 50%. Spondyloarthritis, previously regarded as a male predominant phenomenon, is now considered in some of its forms as having equal distribution between the sexes. As women usually report worse outcome despite better radiographic imaging, the question of comorbid FMS is important. In the study of 103 spondyloarthritis patients, female proportion in the FMS group was substantially higher than non-FMS spondyloarthritis patients (66.7 and 29.4%, respectively). Women also presented with more peripheral vs. axial involvement (27.3 and 14.8%, respectively) [43].

Comorbid FMS may have a significant impact on the comprehension of symptoms severity reported by patients, particularly regarding the degree of fatigue and pain and turns the differentiation of FMS symptoms from an increased rheumatic disease activity challenging.

Cardiovascular comorbidities

On the basis of data from the Taiwanese national health registry, two studies found hazard ratios ranging from 1.47 to –2.11 for coronary heart disease among patients with FMS. The group from Taichung identified 61 612 FMS patients and matched them to 184 834 controls. In a 12-year follow-up, they found FMS to be an independent risk for coronary heart disease, with an increased risk of 47%. They also identified a higher prevalence of traditional risk factors such as hypertension (21.3 vs. 16.1%), hyperlipidemia (14.8 vs. 8.9%), diabetes mellitus (9.2 vs. 6.9%), cerebrovascular diseases (7.8 vs. 5.2%) and congestive heart failure (CHF) (1.1 vs. 0.9%) among patients with FMS [49,50▪].

A database study of 47 000 Taiwanese patients with FMS and 189 000 controls showed a higher risk of ischemic stroke in patients with FMS by an adjusted hazard ratio of 1.3. Once again, conventional risk factors and comorbidities such as diabetes, hypertension, hyperlipidemia and ischemic heart disease were observed more often in the FMS group. FMS patients exhibited a higher risk of stroke regardless of comorbidities. An important finding was that FMS patients exhibited a higher risk for stroke especially at younger ages (less than 35 years). The researchers concluded that FMS is an independent risk factor for stroke, with a greater effect on younger individuals. Similar to other studies, the authors reached a conclusion that measures preventing stroke should be considered in this high-risk group [51,52].

Other reports underlining the coexistence of aberrant autonomic regulation have been described with FMS, such as increased cardiac sympathetic activity at rest, increased sympathetic activity in stressful conditions, reduced parasympathetic activity, aberrant stroke volume, ECG RR interval, heart rate variability and baroreflex sensitivity [53–57].

Vascular injury has also been mentioned in patients with FMS, including increased carotid intima media thickness, impaired aortic elasticity with a larger diameter and degree of arterial stiffness (especially in the presence of antibodies to thyroperoxidase) and a higher fibronectin concentration (reflecting vascular injury). Yet, most researchers agree that further investigation is warranted to clarify the significance of these findings [58–62].

Mitral valve prolapse has also been linked to FMS. Turkish investigators observed a 20% rate (15 of 75 female patients), as well as a nine-fold increased rate of mitral valve prolapse in a group of patients with benign joint hypermobility syndrome [63].

Diabetes mellitus

Most of the literature to date shows a high frequency of FMS in diabetes mellitus patients, ranging from 9 to 23.3% [34,64,65]. Patients with diabetes mellitus with comorbid FMS have more tender points and more complaints of widespread pain than diabetes mellitus without FMS or RA controls [66]. Italian researchers even suggested that insulin resistance might be a risk factor for memory deficit among patients with FMS through sympathetic nervous system activation and hypothalamic pituitary axis dysregulation [67].

Peripheral neuropathy is a common complication of diabetes mellitus. In a study of 100 type two diabetes mellitus patients in Saudi Arabia, peripheral neuropathy was found in 61.9% of patients with diabetes mellitus and coexistent FMS compared

with only 2.5% in diabetes mellitus patients without this comorbidity [64].

Gastrointestinal comorbidities

Irritable bowel syndrome

Previous studies have pointed out the relationship between gastrointestinal disorders and FMS. Most of the literature deals with the association between FMS and functional gastrointestinal disorders and specifically with the irritable bowel syndrome (IBS). The prevalence of IBS ranges from 12.9 to 31.6% in different studies [34].

As the Rome diagnostic criteria include IBS as part of the Functional Gastrointestinal Disorders, characteristic symptoms often include abdominal discomfort or pain, abnormal defecation, bloating, urgency, and so on, complaints that are often encountered in patients with FMS [68–71]. Therefore, several previous studies reported IBS in 30–81% of FMS patients [69,71]. In accordance, studies have also demonstrated higher frequency of FMS amongst IBS patients [34,42]

A study from Oslo investigated a group of patients with perceived food hypersensitivity and found IBS in almost all patients. Extraintestinal symptoms suggestive of FMS (IBS, musculoskeletal pain and chronic fatigue) were found in 71% of these patients [71]. Slim *et al.* [70] summarized 19 clinical studies of gastrointestinal symptoms in FMS, almost all of which demonstrated significantly higher rates of IBS among FMS patients.

In a national prospective cohort study, Yang *et al.* [69] compared 33 729 FMS patients to 134 915 controls and found 1.54-fold increased risk for IBS and 1.67-fold risk when the FMS patient had any additional comorbidity. They also showed that certain medications such as tramadol had a protective effect against developing IBS.

Celiac disease and gluten-sensitivity

It has been found that celiac disease and gluten-sensitivity are prevalent in patients with FMS [72,73▪,74]. A Spanish group reported that gastrointestinal symptoms were more common among FMS patients than gluten-sensitive patients [72]. Despite this finding, screening for celiac disease in patients with FMS is discouraged by many researchers [73▪,74,75].

Inflammatory bowel disease

Most of the research regarding extraintestinal manifestations of inflammatory bowel disease (IBD, Crohn's disease and ulcerative colitis) does not include FMS. However, two studies addressed this issue and found disparate results regarding the rate of FMS in IBD patients. The disparities between the observations of Palm *et al.* [76] and Buskila *et al.* [77] were FMS in 3.0 and 49% in patients with Crohn's disease and 3.7 and 19% in those with ulcerative colitis, respectively. Further studies are required to achieve consist results regarding FMS prevalence in IBD patients.

Cancer and fibromyalgia syndrome

Cancer is a known risk factor for pain, depression, anxiety, fatigue, sleep disturbances, headaches and overall decreased quality of life; symptoms that overlap with FMS [78–83]. However, reports dealing with the association of cancer and FMS are limited and mostly involve breast cancer. Israeli researchers sought to assess FMS in patients with nonmetastatic breast cancer and controls. FMS ACR criteria, tenderness, FMS severity, pain, disability and psychiatric comorbidities score were assessed. Although FMS was found in only four women (5%) due to insufficient number of tender points, FMS characteristics were significantly higher in the cancer patient group; 27.5% described constant widespread pain as opposed to only 7.5% of controls; 60% of patients reported sleep disturbances, whereas only 35% of controls did. An association between patients' affective condition (anxiety, depression) and pain interpretation was seen as well. Perception of pain, to some surprise, was not found to be related to cancer stage, age or marital status [78].

This exemplifies how FMS diagnoses in cancer patients can be tricky. For example, false-positive results can be obtained due to chemotherapy or radiotherapy-related side effects such as oral ulcers, change in taste, dry mouth; neuropathies; ototoxicity resulting in hearing problems and dizziness; interstitial lung injury causing chest pain; and skin and vascular injuries due to Raynaud's syndrome, photosensitivity, among others. On the contrary, underreporting may also occur due to attributing vague symptoms of FMS and depression to cancer treatment. Surgical procedures such as colostomy or any abdominal changes postoperatively (e.g. adhesions) can cause IBS and make the diagnosis of FMS troublesome [83].

These data clearly indicate that physicians treating cancer patients at all stages should be familiar with FMS symptomatology, which would lead to FMS diagnoses and therapy that are more accurate. On the contrary, symptoms present in a known FMS patient could mask a malignancy and thus delay diagnosis.

Table 1. Comorbidities of specific interest in the fibromyalgia syndrome

Psychiatric [15–22,23▪,24,25▪,26–31]	Rheumatologic [32–39,40▪,41–48]	Cardiovascular [49,50▪,51–67]	Gastrointestinal [68–72,73▪,74–77]	Others
PTSD [11,23▪,24,26,27] Depressive disorder [15–18,20,27,28,30,31,47] Anxiety disorder [17,27,28,30,31] Bipolar disorder [18,19,21,25▪,29] Personality disorder [19,22]	RA [32,38,44,47,48] SLE [32,37,44] Ankylosing spondylitis [44] Osteoarthritis [44] Familial Mediterranean fever [44] Behçet's disease [44] Gout [44] Sjögren's syndrome [39,42,44,46] Vasculitis [44] Polymyalgia rheumatica [44] Polymyositis [44] Spondyloarthritis [43] Photosensitivity [30,44] Raynaud's phenomena [30,44] ANA positivity [30]	Coronary heart disease [49,50▪] Hypertension [49,50▪] Hyperlipidemia [49,50▪] Diabetes mellitus [34,49,50▪,64–67] Cerebrovascular diseases [49,50▪] CHF [49,50▪] Ischemic stroke [51,52] Aberrant autonomic regulation [53–57] Vascular injury [58–62] Mitral valve prolapse [63]	Irritable bowel syndrome [34,42,68–71] Celiac disease and Gluten-sensitivity [72,73▪,74,75] Crohn's disease [76,77] Ulcerative colitis [76,77]	Peripheral neuropathy [30] Cancer [78–83]

CONCLUSION

FMS is a common finding, especially with psychiatric or rheumatologic disorders. Consequently, physicians should aim for early recognition of comorbid illnesses, which may lead to better adherence on the part of patients and management of treatment by healthcare staff of both FMS and the comorbid illness (Table 1).

Acknowledgements

None.

Financial support and sponsorship

None.

Conflicts of interest

This work was performed in partial fulfilment of the M.D. thesis requirements of the Sackler Faculty of Medicine, Tel Aviv University, Israel (A.L).

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Targeting aging for disease modification in osteoarthritis

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Purpose of review
Age is a key risk factor for the development of osteoarthritis and age-related changes within the joint might represent targets for therapy. The recent literature was reviewed to find studies that provide new insight into the role of aging in osteoarthritis, with a focus on the potential for disease modification.

Recent findings
Preclinical studies using isolated cells and animal models provide evidence that two hallmarks of aging (cellular senescence and mitochondrial dysfunction) contribute to the development of osteoarthritis. Senescent cells secrete pro-inflammatory mediators and matrix degrading enzymes, and killing these cells with 'senolytic' compounds has emerged as a potential disease-modifying therapy. Mitochondrial dysfunction is associated with increased levels of reactive oxygen species (ROS) that can promote osteoarthritis by disrupting homeostatic intracellular signaling. Reducing ROS production in the mitochondria, stimulating antioxidant gene expression through Nrf2 activation, or inhibiting specific redox-sensitive signaling proteins represent additional approaches to disease modification in osteoarthritis that require further investigation.

Summary
Although no human clinical trials for osteoarthritis have specifically targeted aging, preclinical studies suggest that targeting cellular senescence and/or mitochondrial dysfunction and the effects of excessive ROS may lead to novel interventions that could slow the progression of osteoarthritis.

Keywords
aging, cell senescence, cell signaling, mitochondria, reactive oxygen species

INTRODUCTION

Osteoarthritis is one of the most common causes of pain and disability in older adults [1,2]. Management of osteoarthritis is limited to symptomatic treatments that many people with osteoarthritis find inadequate and there is a lack of any intervention proven to alter the natural course of OA. With the rapidly growing numbers of older adults in the United States and other developed countries, there is a significant need for research that will lead to new interventions that target both symptoms and disease progression.

It is well accepted that increasing age is a major risk factor for osteoarthritis and there is a growing body of evidence that aging processes within the joint, and perhaps systemically as well, contribute to the development and progression of osteoarthritis [3]. Therefore, a better understanding of how aging and osteoarthritis interrelate could lead to new targets or strategies for intervention. Geroscientists have suggested that similar age-related processes may contribute to multiple conditions associated with aging such as cardiovascular disease, cancer, neurocognitive disorders and musculoskeletal conditions [4]. Hallmarks of aging have been identified that represent the processes most likely to contribute to age-related conditions and include stem cell exhaustion, altered intercellular communication, genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, cellular senescence, and mitochondrial dysfunction [5]. For the purposes of this review,

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Curr Opin Rheumatol 2018, 30:101–107

DOI:10.1097/BOR.0000000000000456

KEY POINTS

- There is mounting evidence that cellular senescence and mitochondrial dysfunction, two of the hallmarks of aging, contribute to the development of osteoarthritis.
- Targeting cellular senescence in joint tissues by killing senescent cells has been shown in a preclinical study in mice to reduce the severity of age-related osteoarthritis, and osteoarthritis induced by ACLT.
- Mitochondrial dysfunction results in increased levels of ROS that disrupt homeostatic cell signaling through protein thiol oxidation.
- Increasing the antioxidant capacity of joint tissue cells through transgenic overexpression of catalase targeted to the mitochondria reduced the severity of age-related osteoarthritis in mice. Treatment with a small molecule mitochondrial antioxidant, MitoTempo, reduced the severity of osteoarthritis in rats on a high cholesterol diet that also underwent DMM surgery to induce osteoarthritis.
- Further work is necessary to move promising approaches that alter aging processes in osteoarthritis from preclinical models to humans, including identifying the tissues and particular forms of osteoarthritis that should be targeted.

we will focus on the latter two, cellular senescence and mitochondrial dysfunction with its accompaniment of oxidative stress, where recent progress has been made that is contributing to the knowledge of mechanisms linking aging and osteoarthritis. We will include a review of preclinical studies that suggest targeting these age-related processes could be disease modifying.

CELLULAR SENESCENCE AND OSTEOARTHRITIS

Cellular senescence is a response to persistent stress that can be characterized by stable cell-cycle arrest, increased expression of the cell cycle inhibitor $p16^{Ink4a}$, and enhanced production of inflammatory cytokines and other factors known as the senescence-associated secretory phenotype (SASP) [6]. It has been well known for decades that cellular senescence occurs during the extended culture of cells in monolayer. The phenomenon of in-vitro senescence does have relevance to osteoarthritis therapies because it limits the capacity of patient-derived chondrocytes to be expanded for regenerative medicine applications [7]. There is also substantial evidence that cellular senescence develops with aging *in vivo* and plays an important role in driving age-related disease [8]. Recent developments in the ability to selectively induce apoptosis of senescent cells from within tissues have advanced basic science and generated excitement about the therapeutic potential of targeting senescent cells [9].

DEVELOPMENT OF THE 'SENOLYTIC' STRATEGY

The rationale for eliminating senescent cells was enhanced by studies with 'INK-ATTAC' mice in which senescent cells are killed by a tailored drug through expression of a transgene that is only expressed in cells with high levels of $p16^{Ink4a}$ [10▪,11]. Although the effect on osteoarthritis was not reported, a reduction in specific age-related pathologies occurred in both progeroid and naturally aged models [10▪,11]. The next development was to identify small molecules that achieve the same effects without requiring transgenic mice. One strategy for developing these 'senolytic' compounds has been to inhibit pathways that are upregulated in senescent cells, which is akin to the approach commonly used in developing chemotherapeutics. For example, the senolytic Navitoclax (ABT-263) inhibits Bcl-2 and Bcl-xL, which are part of the antiapoptotic machinery that allows senescent cells to survive in the context of persistent stress [12]. Delivery of Navitoclax was shown to specifically kill senescent cells *in vitro* and *in vivo*, promoting enhanced function of the remaining stem cells in the hematopoietic system and muscle [13].

THE RATIONALE FOR OSTEOARTHRITIS AS A POTENTIAL TARGET FOR SENOLYTIC THERAPY

The potential role for senescence during the pathogenesis of osteoarthritis has recently been reviewed [3,14], but published investigations have relied heavily on correlative evidence that cells with specific markers of senescence emerge with age and osteoarthritis. A recent example showed that osteoarthritis disease severity correlates with senescence-associated beta galactosidase activity [15▪], a commonly used marker of senescence that represents high lysosomal activity. An alternative approach evaluated the general concept that exogenous cells secreting a potent SASP can drive osteoarthritis. In this study, delivery of irradiation-induced senescent ear fibroblasts directly to the joint caused cartilage degradation as compared to control cells [16▪].

Senolytics provide a further opportunity to help determine the functional role of senescence by targeting senescent cells that arise spontaneously with age or in response to acute stress. Inducing apoptosis

of chondrocytes may seem like a risky strategy given that articular chondrocytes are responsible for producing and maintaining the extracellular matrix. However, recent data demonstrate that killing chondrocytes that would otherwise secrete matrix-degrading factors such as matrix metalloproteinase 13 (MMP13) can protect the tissue in a posttraumatic osteoarthritis model [17▪]. The tolerance for chondrocyte death in murine models may be partly because of the high cell density found within the cartilage of mice, and it remains to be seen whether the lower cell density in human cartilage alters the capability to withstand cell loss. The concept of inducing cell death during aging may also be feasible given the extraordinary stability of aggrecan and type II collagen [18], as maintaining the capacity for new matrix synthesis with aging may not be as essential as ensuring protection from matrix degrading enzymes that are produced by senescent cells. The potential negative effects of eliminating senescent cells (and potentially off-target cells) must be taken seriously for osteoarthritis, as the slow course of disease progression may necessitate periodic treatment over years to have the maximal therapeutic effect.

INTRA-ARTICULAR DELIVERY OF A NOVEL SENOLYTIC REDUCES POSTTRAUMATIC OSTEOARTHRITIS

Initial evidence that senolytics may improve the function of cartilaginous tissues came from the observation that weekly treatment with the senolytic combination of dasatinib and quercetin resulted in a higher glycosaminoglycan content in the intervertebral disc [19]. The direct application of a senolytic approach for osteoarthritis was explored using anterior cruciate ligament transection (ACLT) in both a transgenic model (p16–3MR mice in which p16-high cells are eliminated with ganciclovir) and using a proprietary senolytic compound that had not been previously described (UBX0101) [20▪▪]. With repeated intra-articular injections beginning two weeks after ACLT, UBX0101 reduced the burden of senescent chondrocytes and production of SASP factors. The treatments also limited the development of osteoarthritis by histological analysis and improved the function of the injured joint as assessed by weight distribution. Inducing death of senescent cells showed a benefit despite the calculated intra-articular half-life of 1.5 h, which illustrates that this approach may have advantages over osteoarthritis therapies that require continuous biologic activity to mediate the intended effects. The authors also show protection from osteoarthritis in a small number of aged animals using senescence clearance in the previously described INK-ATTAC model, raising the exciting possibility that senescence may be a valid target for age-related osteoarthritis in addition to the posttraumatic setting [20▪▪]. Finally, the authors provided evidence that UBX0101 targets senescent human chondrocytes using a pellet culture model, with the surrounding cells compensating for the death of senescent cells by enhanced proliferation and matrix synthesis.

REDOX SIGNALING PATHWAYS AS THERAPEUTIC TARGETS IN OSTEOARTHRITIS

It is becoming widely accepted that age-related increases in reactive oxygen species (ROS) levels, combined with a reduced intracellular antioxidant capacity, leads to oxidative stress-induced cellular damage that contributes to the progression of age-related diseases, including osteoarthritis [21]. The specific mechanisms linking oxidative stress to disease, however, remain poorly understood. Although some theories of aging suggest that oxidative stress contributes to age-related disease through random cellular damage, more recent theories align themselves with the concept that age-related oxidative stress leads to cellular dysfunction through disturbing normal homeostatic cell signaling pathways [21,22].

CYSTEINE SULFENYLATION REGULATES CHONDROCYTE SIGNALING

One proposed key mechanism by which ROS regulate cell signaling is through posttranslational oxidation of reactive cysteines by H_2O_2 (protein thiol oxidation). Thiol oxidation results in the formation of a cysteine sulfenic acid (Cys-SOH) termed S-sulfenylation, which can lead to either oxidative inhibition or activation of protein function that is dependent on the properties of the specific protein [23]. Recent data from Wood *et al.* [24] demonstrate that chondrocytes derived from osteoarthritis cartilage display increased levels of basal S-sulfenylation when compared to chondrocytes derived from non-osteoarthritis cartilage. Treatment with fragments of fibronectin (FN-f) to induce physiological levels of H_2O_2 led to sulfenylation of the tyrosine-protein kinase Src (Src), which enhanced Src activity and MMP-13 production. Pretreatment with dimedone, to block sulfenylation, or N-acetyl cysteine, to reduce levels of ROS, decreased Src sulfenylation and abrogated these effects. These data suggest that sulfenylation of cartilage proteins can significantly contribute to joint degradation by regulating redox-signaling events. Strategies aimed at reducing protein sulfenylation, either directly or

through targeted antioxidant treatment, could restore homeostatic signaling to protect against osteoarthritis.

In contrast to physiologic levels of H_2O_2, excessive levels can hyperoxidize cysteines to form sulfinic acid (Cys-SO_2H) or sulfonic acid (Cys-SO_3H). Hyperoxidation is most often irreversible and leads to inhibition of protein function, as has been observed with hyperoxidation of the peroxiredoxin (Prx) family of peroxidase enzymes [25].

MITOCHONDRIAL DYSFUNCTION AND REDOX SIGNALING IN OSTEOARTHRITIS

The mitochondrion is a key source of ROS. Mitochondrial function has been shown to decline with age and a causative link between age-related mitochondrial dysfunction, oxidative stress and disease has been put forth [5]. Mitochondrial Prx3 hyperoxidation was found to be greater in older human chondrocytes when compared to younger chondrocytes, both basally and in response to mitochondrial H_2O_2 induced by the redox cycling oxidant, menadione [26▪]. Menadione-induced Prx3 hyperoxidation was associated with inhibition of prosurvival Akt signaling and activation of p38-mediated cell death. Expression of catalase targeted to the mitochondria (MCAT) abrogated these effects by preventing Prx hyperoxidation and restoring homeostatic signaling to maintain cell viability. Importantly, in an *in vivo* aging model, transgenic MCAT mice displayed reduced severity of age-related osteoarthritis when compared to wild-type mice [26▪]. Similarly, in another recent study, treatment with the mitochondrial targeted antioxidant mitoTEMPO attenuated the severity of cholesterol-induced osteoarthritis in rats and mice [27▪]. Mice deficient in Apolipoprotein E (ApoE) and rats with diet-induced hypercholesterolemia (DIHC) subjected to destabilization of the medial meniscus (DMM) surgery displayed enhanced severity of osteoarthritis symptoms when compared to animals fed a control diet or animals subjected to sham operations. A daily regimen of MitoTEMPO for 5 weeks beginning 1-week after DMM surgery significantly reduced osteoarthritis severity as assessed by the Mankin score. *In vitro* studies showed that MitoTEMPO reduced cholesterol-induced ROS generation, partially restored ATP levels, and decreased MMP13 expression and markers of apoptosis in primary human articular cartilage chondrocyte pellets [27▪].

As further evidence for a role of mitochondrial ROS in osteoarthritis, deletion of superoxide dismutase 2 (Sod2), enhanced the severity of osteoarthritis in mice [28]. This suggests that this mitochondrial antioxidant, which catalyzes the detoxification of mitochondrial superoxide, may also be of importance in the pathogenesis of osteoarthritis. Fu *et al.* [29▪▪] recently conducted an in-depth analysis of the antioxidant network from the cartilage of adult (10 month-old) and aging (30-month-old) rats and showed that, although mitochondrial Sod2 levels in aging rats increased compared with adult rats, specific Sod2 activity was reduced. Sod2 activity is impaired by posttranslational acetylation in many cell types and this process is finely regulated by the mitochondrial deacetylase enzyme sirtuin3 (Sirt3) [30]. Accordingly, reduced Sod2 activity in aging rats was associated with an increase in Sod2 acetylation and a reduction in Sirt3 protein abundance. Incubation of cartilage homogenates with recombinant Sirt3 and its cofactor, NAD^+, led to increased Sod2 enzymatic activity [29▪▪]. This effect was greater in homogenates from aged rats, suggesting that an age-related decline in Sirt3 levels may underlie impaired Sod2 function in aging cartilage. Taken together, these studies suggest that targeting the mitochondria to counter age-associated, oxidative stress-induced disturbances in redox signaling pathways could represent a novel therapeutic strategy to slow or stop the progression of OA.

MITOCHONDRIAL GENETICS INFLUENCE REDOX SENSITIVITY AND OA

In addition to mitochondrial dysfunction, mitochondrial genetics may also play a role in osteoarthritis incidence and progression [31]. Fernández-Moreno *et al.* [32▪] investigated human subjects from the osteoarthritis initiative (OAI) and the cohort hip and cohort knee studies to demonstrate that mitochondrial DNA haplogroup J associates with a reduced risk of incident knee osteoarthritis when compared to mitochondrial DNA haplogroup H. To identify specific differences between these haplogroups, transmitochondrial cybrids harboring haplogroup J or H were constructed and metabolic and redox parameters were analyzed. Haplogroup H cybrids displayed enhanced glycolysis and mitochondrial respiration, which led to increased ATP production when compared to haplogroup J cybrids. Haplogroup H cybrids also exhibited higher production of peroxynitrite, peroxide, and mitochondrial superoxide, and an increased susceptibility to cell death in response to H_2O_2. Thus, it appears that metabolic dysfunction and redox imbalance are closely related to osteoarthritis prevalence. Therapeutic drugs aimed at targeting these specific parameters, or to more closely mimic those of haplogroup J, may be of value in osteoarthritis therapy.

REGULATION OF NUCLEAR RECEPTOR ERYTHROID 2-RELATED FACTOR 2 SIGNALING IN OSTEOARTHRITIS

Nuclear receptor erythroid 2-related factor 2 (Nrf2) represents an important transcription factor that regulates the expression of a wide array of phase II antioxidant genes relevant to maintaining cellular redox homeostasis [33,34]. There is evidence for decline in Nrf2 protein levels in human osteoarthritis chondrocytes when compared to healthy controls [35]. Recent data from Cai *et al.* [36▪] demonstrate that treatment with trichostatin A (TSA), a broad-spectrum histone deacetylase inhibitor (HDACi), reduces the severity of osteoarthritis in both a posttraumatic mouse model (DMM surgery) and an inflammatory mouse model of osteoarthritis [monosodium iodoacetate (MIA) injection]. Analysis of cartilage from the joints of these mice showed that treatment with TSA enhanced acetylation-induced Nrf2 activation and the expression of the Nrf2 antioxidant target genes heme oxygenease-1 (HO-1) and NAD(P)H: quinone oxidoreductase 1 (NQO-1). This was associated with reduced catabolic cytokine production and decreased MMP release. Treatment with TSA was unable to offer protection against DMM and MIA-induced osteoarthritis in Nrf2 knockout mice suggesting that the protective effect of HDACi on osteoarthritis severity was Nrf2 dependent.

In addition, several recent studies have assessed the role of active Nrf2 to regulate chondrocyte inflammatory signaling pathways. For example, Vaamonde-Garcia *et al.* [37] demonstrate that pharmacological activation of Nrf2 or its downstream target, HO-1, leads to a reduction in interleukin (IL)-1β induced ROS generation and IL-6 production in murine chondrocytes. The purported antioxidant compounds Wogonin [38], pterostilbene [39], protandim and 6-gingerol [40] have also been shown to exert chondroprotective effects through ROS-induced activation of the Nrf2 pathway and suppression of inflammatory mediators such as PGE2 and nitric oxide production. Thus, strategies aimed at maintaining active Nrf2 in aging cartilage may hold promise for osteoarthritis therapy but the precise mechanisms underlying Nrf2 regulation in aging human joint tissues requires further characterization.

CONCLUSION AND FUTURE DIRECTION

Advances in the understanding of how senescence and oxidative stress develops in the joint and how this contributes to osteoarthritis will help in the design of more targeted senolytic and antioxidant therapies that could be used to slow or stop osteoarthritis from progressing. One key question related to senolytics is determining if other cell types other than chondrocytes would be important to target. Cells from the synovium and infrapatellar fat pad could potentially secrete SASP factors directly into the joint space and drive the inflammatory cascades that can occur with osteoarthritis. Given the relationship between cartilage and the health of underlying bone in osteoarthritis development, it is of interest that markers of senescence and the SASP also increase with age in osteocytes [41]. Innovative methods to measure multiple indicators of senescence on a single-cell basis [42] may be helpful for quantifying the senescence burden in particular cell types. Indeed, it is feasible that senescence may emerge as a biomarker to help phenotype the particular forms of osteoarthritis and that senolytic therapies would be most effective in a subset of patients [43].

The studies reviewed above provide evidence that oxidative stress can contribute to the development and progression of age-related osteoarthritis through disturbing homeostatic redox signaling pathways. Anabolic and cell survival pathways appear to be more susceptible to inhibition during conditions of oxidative stress whereas catabolic and cell death pathways are more active. This altered balance in signaling pathways may result from attempted degradation and removal of proteins that have been damaged by oxidative stress, with cell death occurring if homeostasis cannot be restored. The design of disease modifying osteoarthritis drugs aimed at maintaining antioxidant signaling pathways in aging, such as those governed by the Prx's, Sod's, Sirt's, and Nrf2, hold novel therapeutic value in the prevention or treatment of osteoarthritis. Such therapeutics will replace the current untargeted antioxidant approach with disease modifying osteoarthritis drugs that target specific signal transduction pathways that contribute to osteoarthritis.

The connection between oxidative stress and the promotion of cellular senescence has not been fully explored in the context of cartilage and osteoarthritis. A recent study in mice with deletion of the antioxidant gene superoxide dismutase found evidence for increased accumulation of senescent cells in the kidney [44]. Future studies investigating whether increased oxidative stress with aging plays a causal role in the development of the senescent phenotype in the joint will be important to designing effective therapeutic strategies. Preventing the emergence of senescence with targeted antioxidant therapies may be one strategy to delay the onset of osteoarthritis before severe matrix degradation and inflammatory pathways are established.

Acknowledgements

None.

Financial support and sponsorship

The research of J.A.C. was supported by the American Federation for Aging Research and the National Institute on Aging (AG044034), B.O.D. was supported by a joint grant from the American Federation for Aging Research and the Arthritis National Research Foundation, and the National Institute on Aging (AG050399), and R.F.L. was supported by the National Institute on Aging (AG044034) and the National Institute of Arthritis, Musculoskeletal, and Skin Disease (AR049003).

Conflicts of interest

R.F.L. is a consultant for Unity Biotechnology. The remaining authors have no conflicts of interest.

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Bioactive lipids in osteoarthritis: risk or benefit?

Andreea Ioan-Facsinay and Margreet Kloppenburg

Purpose of review
Lipids are bioactive molecules that can affect several biological functions. Technological developments allowing identification of novel lipid species and the study of their function have led to a significant advance in our understanding of lipid biology and their involvement in various diseases. This is particularly relevant for diseases associated with obesity in which lipid accumulation could be involved in pathogenesis. Here, we focus on osteoarthritis, a chronic joint disease aggravated by obesity, and will present the latest findings regarding the involvement of lipids in disease development and progression.

Recent findings
Recent studies indicate a possible involvement of n-3 poly-unsaturated fatty acid and their anti-inflammatory and proresolving derivatives in osteoarthritis. These lipids were identified in the osteoarthritis joint, were found to have beneficial effects on cartilage *in vitro* and reduced pain in humans and animal models. Moreover, increased levels of cholesterol transport molecules, such as LDL particles, were recently associated with a higher risk of developing hand osteoarthritis in women and with more severe inflammation and osteophyte formation in osteoarthritis animal models.

Summary
Together, these findings indicate that lipids are a promising target for future therapeutic intervention in osteoarthritis and open exciting possibilities for future research.

Keywords
cholesterol, fatty acids, lipids, osteoarthritis

INTRODUCTION

Since the discovery of the association between obesity and the increased risk for development and progression of osteoarthritis, increasing attention has been given to the identification of bioactive molecules that could explain this association. Obesity is accompanied by low-grade inflammation and increased levels of circulating adipokines, cytokines and lipids, as well as metabolic complications such as metabolic syndrome, cardiovascular complications and diabetes [1]. Both soluble factors and metabolic complications associated with obesity have been implicated in osteoarthritis pathogenesis, but the molecular mechanisms underlying this association are still poorly understood. However, as lipid accumulation is the main characteristic of obesity, a lot of attention has focused on the presence and function of lipids in osteoarthritis. Lipid classes that are elevated in obesity include free fatty acids and cholesterol, as well as higher order lipids involved in transport of lipids in the circulation, such as triglycerides and lipoproteins [2]. Both free fatty acids and cholesterol-containing lipoproteins have been previously implicated in osteoarthritis pathogenesis through their ability to modulate inflammatory responses, as well as cartilage and bone metabolism [3,4]. In the past 18 months, a series of novel findings have been published regarding the presence and possible role of n-3 poly-unsaturated fatty acids (n-3 PUFAs) and their hydroxylated derivatives in osteoarthritis. Moreover, novel insights into the contribution of cholesterol metabolism to osteoarthritis have been obtained. These findings will be placed in the context of previous knowledge and will represent the focus of the present review.

FATTY ACIDS AND HYDROXYLATED DERIVATIVES IN OSTEOARTHRITIS

Fatty acids can have different biological effects, depending on their length and degree of saturation.

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Curr Opin Rheumatol 2018, 30:108–113
DOI:10.1097/BOR.0000000000000463

KEY POINTS

- Anti-inflammatory and proresolving lipid mediators derived from n-3 PUFA are present in the osteoarthritis joint, but are not able to completely resolve inflammation.
- D-series resolvins have anti-inflammatory and antiapoptotic effects on cartilage.
- N-3 PUFA and D-series resolvins reduce osteoarthritis-associated pain in humans and animal models.
- High LDL is associated with increased development of hand osteoarthritis in women and increased synovitis and osteophyte formation in animal models.

In general, saturated fatty acids are believed to be predominantly proinflammatory, whereas unsaturated fatty acids have more anti-inflammatory functions. Within PUFAs, one can distinguish between the more proinflammatory n-6 PUFA such as linoleic acid and arachidonic acid and the rather anti-inflammatory n-3 PUFA, such as alpha-linoleic acid, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). N-6 PUFA can be further oxidized into bioactive metabolites such as the proinflammatory prostaglandins and leukotrienes, whereas n-3 PUFA can be oxidized to specialized proresolving mediators (SPMs) and other oxylipins with potent anti-inflammatory and proresolving functions [5].

Prostaglandins and leukotrienes are the best studied in the context of osteoarthritis and have been shown to be present in plasma and synovial fluid of osteoarthritis patients and to have proinflammatory and catabolic effects on fibroblasts, osteoblasts and cartilage [3]. Moreover, prostaglandin E2 (PGE2) and 15-HETE (an oxylipin derived from arachidonic acid) levels were associated with the presence of knee osteoarthritis [6], indicating a possible role for these oxylipins in osteoarthritis development.

Other oxylipins, such as those derived from the n-3 PUFA DHA and EPA, have received much less attention in osteoarthritis. The main functions ascribed to n-3 PUFA and SPM include inhibition of proinflammatory cytokine production, inhibition of neutrophil migration, enhancement of noninflammatory removal of dead/apoptotic cells, enhanced wound healing, decreased pain and others [7]. Because the osteoarthritic process resembles in several aspects a chronic wound accompanied by cell death, inflammation and pain [8] and because n-3 PUFA/SPM have been shown to target all these processes, it is conceivable that these lipids could be effective therapeutic agents in osteoarthritis.

In the context of osteoarthritis, only few studies have investigated the fatty acids present in osteoarthritis patients and their relationship to clinical manifestations. These studies indicated that increased n-3 fatty acids were associated with less patellofemoral cartilage loss and n-6 fatty acids with increased synovitis at 30 months follow-up in 472 individuals participating in the MOST study, a cohort of osteoarthritis patients and individuals at risk of osteoarthritis development [9]. These findings were strengthened by a more recent study, in which a randomized double-blind study was performed in which 202 patients with knee osteoarthritis (cf. American College of Rheumatology criteria) and having knee pain (visual analogue scale pain >20 mm) were treated with either high-dose (4,5 g/day n-3 fatty acids) or low-dose (0,45 g/day n-3 fatty acids combined with sunola oil) fish oil for 24 months [10▪]. A significant change in WOMAC pain and function was observed in both groups at 24 months and this change was more pronnounced in the low-dose group [high dose – low dose WOMAC at 24 months (SE): 3.3 (1.3)]. These results are promising, but have to interpreted with caution, as the study was not placebo-controlled and the low-dose group has also received sunola oil, which could explain the improved pain scores. There were no significant changes observed in cartilage loss, nor any improvement in bone mineral density after 24 months in either group [11].

These data were supported by another randomized double-blind placebo-controlled study in osteoarthritic dogs showing that administration of 69 mg/kg/day of triglycerides n-3 oil (containing DHA and EPA) improved all measures of pain, discomfort and function after 84 days of treatment compared to the placebo group [12]. In both studies, the levels of n-3 PUFA in blood were increased in the treated groups, indicating compliance.

All studies performed with n-3 PUFA suggest that the beneficial effects are primarily an improvement in pain and symptoms, whereas little effect is observed on structural progression of the disease. However, previous studies have indicated that n-3 PUFA can counteract the proinflammatory and catabolic actions of interleukin-1α (IL-1α) on cartilage *in vitro* [13]. These results were complemented by a new study in which the authors show that DHA downregulates MMP-13 through a P38 mitogen-activated protein kinases (p38-MAPK)-mediated mechanism [14] both *in vitro* and *in vivo* in a rat model of osteoarthritis.

Apart from direct effects of n-3 PUFA on osteoarthritis joints, it is conceivable that n-3-derived oxylipins could be generated *in vitro* and these could (at least partially) mediate the observed effects.

Metabolites of n-3 PUFA have been previously described in rheumatoid arthritis (RA) synovial fluid [15] and in a more recent study also in osteoarthritis synovial fluid. In this observational study, we have employed a targeted lipidomic approach to investigate the presence of both proinflammatory and anti-inflammatory/proresolving lipids in synovial fluid of 24 osteoarthritis patients compared to 10 RA patients [16▪▪]. Our data confirmed that proinflammatory lipid mediators, such as PGE2, can be found in osteoarthritis synovial fluid and identified for the first time oxylipins derived from n-3 and n-6 PUFA that are biomarkers of resolution and precursors of specialized proresolving lipid mediators. These include 15-HETE (derived from arachidonic acid), as well as 17-HDHA (derived from DHA) and 18-HEPE (derived from EPA). In the unfractionated synovial fluid, the SPM resolvin D2 (RvD2) could be detected as well, indicating that resolution is activated in osteoarthritis patients, albeit insufficiently, as it is not able to completely resolve inflammation. Moreover, we found a similar activity of COX-2 in osteoarthritis and RA, but a lower activity of 5-lipoxygenase and 15-lipoxygenase in osteoarthritis compared to RA. Analysis of synovial fluid cells and synovial cells showed that these are able to produce most of the lipid mediators found in osteoarthritis, except for RvD2. Its cellular source remains to be determined in future studies.

The potential relevance of RvD2 in osteoarthritis was further substantiated in a preclinical study in which the effect of 17-HDHA on osteoarthritis was investigated in two osteoarthritis models in rats: the inflammatory monosodium iodoacetate model and the joint destabilization model of medial meniscal transection (MNX) [17▪▪]. In both models, pain was reduced upon administration of 17-HDHA, whereas discontinuation of treatment in the MNX model restored pain. 17-HDHA administration was accompanied by an increase in systemic levels of RvD1 and RvD2, possibly indicating further oxidation of 17-HDHA to downstream D-series resolvins. Interestingly, the receptor for RvD1 in mice, ALX, was expressed at similar levels both in normal and osteoarthritis joints and was upregulated in dorsal horn of the lumbar spinal cord in rats with osteoarthritis joints at early time points after disease induction, possibly mediating the analgesic effects of RvD1. No effect on synovial inflammation or osteoarthritis-associated structural damage was observed in this study. Confirming and expanding these data, a recent study in 250 sera randomly selected from a population-based twin cohort of 2500 individuals showed that 17-HDHA was significantly associated with higher heat pain threshold and heat pain super threshold [18]. Moreover, higher plasma 17-HDHA was also associated with lower WOMAC pain scores in osteoarthritis patients (62 osteoarthritis patients), independently of the severity of radiographic damage and independently of circulating DHA or total n-3 levels. Collectively, these data indicate that 17-HDHA is involved in pain perception.

Another recent study has shown beneficial effects of resolvin D1 on osteoarthritis chondrocytes. RvD1 belongs to the family of D-series resolvins which includes RvD2–RvD6 and share the common precursor 17-HDHA. In one of the recent studies, RvD1 was shown to inhibit the IL-1β-mediated upregulation of COX-2, PGE2, MMP13 and nitric oxide and prevented chemically induced apoptosis in human osteoarthritis chondrocytes [19]. These effects were mediated through downregulation of nuclear factor kappa-light-chain-enhancer of activated B cells, p38-MAPK and c-Jun N-terminal kinases activation, as well as inactivation of caspase-9 and upregulation of Bcl-2 and Akt. Despite the high concentrations of RvD1 used in this study (μM range), these data indicate for the first time the potency of an SPM to counteract deleterious processes in osteoarthritis chondrocytes.

Other saturated and mono-unsaturated fatty acids have also been studied for their effect on osteoarthritis chondrocytes [20] and fibroblasts [21]. These studies indicate that both saturated and nonsaturated fatty acids have proinflammatory effects on fibroblasts, whereas the effects on chondrocytes vary depending on the procatabolic stimulus used. Three recent studies have expanded these findings and showed that fatty acids, especially the saturated fatty acid palmitate, induce apoptosis in articular chondrocytes [22,23] and meniscal chondrocytes from osteoarthritis patients [24]. The first study used a mixture of palmitate and oleate and showed that it induces reactive oxygen species (ROS), as well as IL-6 and IL-8 in chondrocytes from healthy cartilage. The second study found that this mixture of free fatty acids induces ROS also in osteoarthritis chondrocytes. This was partially due to upregulation of NOX4 and was associated with chondrocyte apoptosis and activation of caspase-3 expression. The third study investigated palmitate and oleate separately and found, similar to the second study, an increased activation of caspase-3 and apoptosis by palmitate, not by oleate. In addition, the authors showed upregulation of proteins involved in endoplasmic reticulum (ER) stress, such as CHOP, and increased signaling of inositol-requiring enzyme 1α, involved in ER stress-induced apoptosis. Regardless of the underlying mechanisms, the data from these studies support a model in which increased free saturated fatty acid levels associated with obesity can mediate chondrocyte death

thereby contributing to osteoarthritis pathogenesis. Whether fatty acids associated with anti-inflammatory effects, such as PUFA or SPM, could counteract the effects of palmitate, remains an interesting question for the future. However, the recent finding that RvD1 could suppress chondrocyte apoptosis [19] raises the interesting possibility that (nutritional) interventions aimed at increasing the relative abundance of PUFA or their derivatives could be beneficial for restoring cartilage homeostasis in osteoarthritis. Moreover, the evidence presented above pointing to a beneficial role for N-3 PUFA and SPM in osteoarthritis, in combination with the knowledge that several biological pathways involved for osteoarthritis pathogenesis can be affected by n-3 PUFA and SPM, opens novel and exiting avenues of research aimed at understanding the full potential of these molecules as therapeutic agents targeting multiple tissues in osteoarthritis. Possible mechanisms engaged by n-3 PUFA and SPM in osteoarthritis are depicted in Fig. 1.

CHOLESTEROL METABOLISM IN OSTEOARTHRITIS

Evidence for a contribution of high cholesterol levels to osteoarthritis development and progression comes primarily from animal studies, whereas only few observational studies were performed in humans. In mice, a diet enriched in cholesterol in combination with a genetic background allowing for an atherogenic lipid profile (high LDL and very low density lipoprotein cholesterol) has been shown to induce [25] or aggravate osteoarthritis [26]. High cholesterol serum levels and atherosclerotic features [25], or accumulation of ApoB (component of LDL particles) in synovial macrophages and their activation leading to more active transforming growth factor beta and increased osteophyte formation [26] were proposed to mediate these effects.

These findings were recently expanded in a study using Apoe−/− mice. These mice display signs of synovial inflammation such as increased S100A8 and A9 expression in synovium and synovial

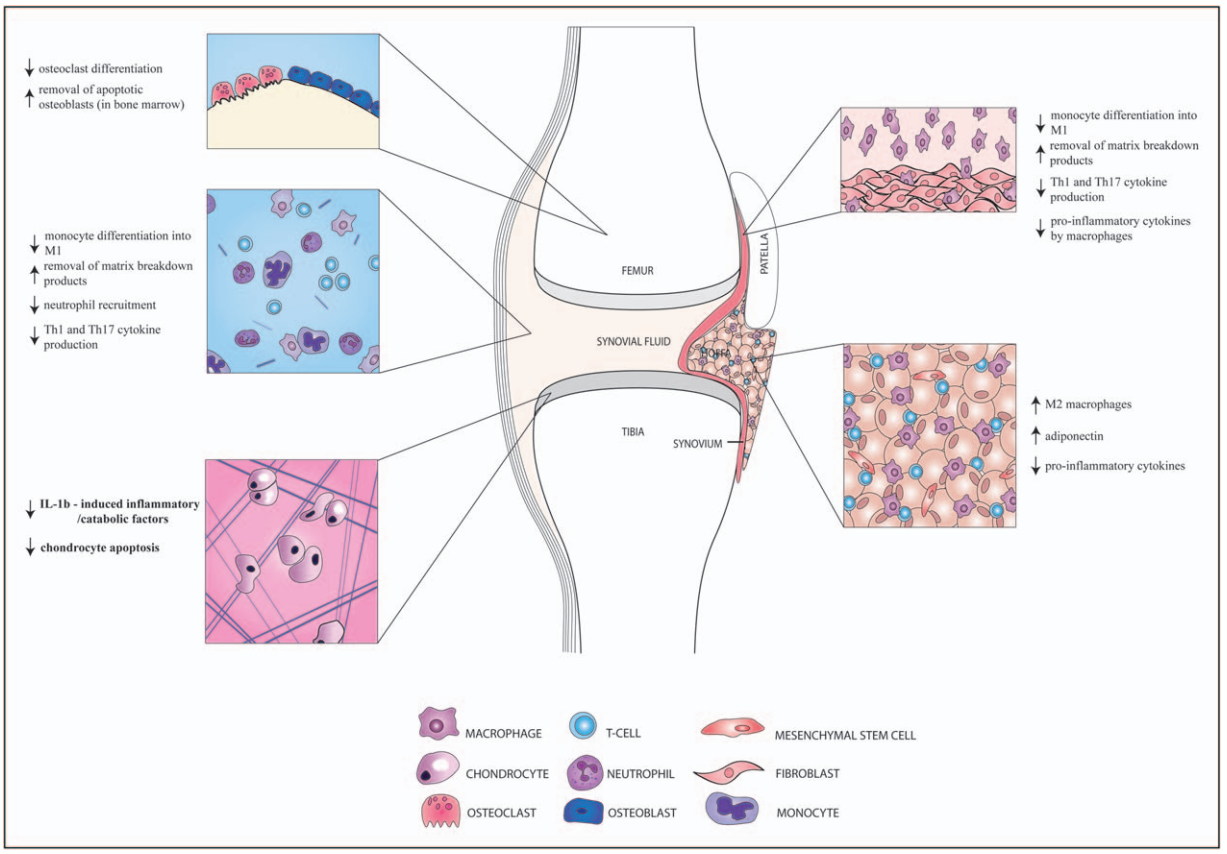

FIGURE 1. Possible effects of n-3 PUFA and SPM on tissues involved in osteoarthritis. Main tissues involved in osteoarthritis: bone, cartilage, synovium, Hoffa's fat pad (or infrapatellar fat pad, IFP) and synovial fluid and their cellular components are depicted. The known effects of n-3 PUFA and SPM on OA tissues are depicted in bold. The potential effects of n-3 PUFA and SPM on tissues involved in OA, based on published data with similar cells and tissues in other inflammatory conditions, are also depicted (regular characters). This work is a derivative of Figure 1 in *Arthritis Research & Therapy*, 2013;15(6):225 by Ioan-Facsinay and Kloppenburg, used under CC BY. n-3 PUFA, n-3 poly-unsaturated fatty acid; OA, osteoarthritis; SPM, specialized proresolving mediator.

thickening upon osteoarthritis induction even on a normal diet [27▪]. These features were associated with increased cartilage damage ($P = 0.013$ for lateral femur) and ectopic bone formation ($P < 0.001$ for medial collateral ligaments) compared with wild-type mice. Inflammation was also higher in Apoe−/− mice on high cholesterol diet ($P < 0.001$ for early timepoints), but no differences were observed in cartilage damage or osteophyte formation compared to wild-type mice, as the latter also developed cartilage damage and ectopic bone formation. However, ectopic bone formation was accelerated in Apoe−/− mice (already at day 10) compared to wild-type controls and was associated with earlier upregulation of S100A8/A9 in synovium. Expression of S100A9 mediated partially the ectopic bone formation in wild-type mice on high cholesterol diet.

In another study using Apoe−/− mice and rats, experimental osteoarthritis was induced by surgical destabilization of the joints [28]. In both models, high cholesterol diet aggravated osteoarthritis severity compared to control chow diet and this was attenuated by the cholesterol-lowering drug atorvastatin. In-vitro and in-vivo studies indicated that cholesterol induces cartilage degradation and chondrocyte hypertrophy, as well as mitochondrial dysfunction indicated by increased ROS and decreased Adenosine triphosphate production, mitochondrial fragmentation, depolarization of mitochondrial membrane and chondrocyte death. Remarkably, treatment with a mitochondrial antioxidant reduced cartilage degradation and chondrocyte death in Apoe−/− animals and restored mitochondrial function *in vitro*. Collectively, these data indicate a direct effect of high cholesterol on chondrocyte function and viability as another possible mechanism, besides synovial inflammation, which could mediate development/progression of osteoarthritis in high cholesterol models.

Only few human studies investigated the relationship between high (LDL) cholesterol and osteoarthritis and they indicated that higher total cholesterol and LDL cholesterol levels are present in serum of osteoarthritis patients compared to controls [29].

Two recent studies have investigated atherogenic lipid profiles and their association with hand osteoarthritis. In the longitudinal Chingford women's study [30], it was shown that although no significant differences in baseline serum lipid profiles were present between participants who did and the ones who did not develop hand osteoarthritis over 11 year follow-up, low high density lipoprotein cholesterol levels were inversely associated with the chance to develop osteoarthritis [$n = 277$, odds ratio (OR) 0.76, 95% confidence interval (CI) 0.42–1.39]. A trend was observed for triglyceride levels, whereas no associations were observed for LDL cholesterol. In another population-based case–control study comprising approximately 20 000 cases and controls, hyperlipidaemia was associated with the presence of hand osteoarthritis (OR 1.37, 95% CI 1.28–1.47) [31]. However, it is unclear from this study whether hypercholesterolemia in particular was also associated with hand osteoarthritis.

Together, these data indicate that atherogenic lipid profiles associate with an increased chance of developing hand osteoarthritis in women. Studies in men are still scarce and preclude a firm conclusion. Moreover, there is yet no conclusive evidence that increased LDL or oxidized LDL levels are associated with osteoarthritis in humans, despite the increasing evidence in preclinical models.

CONCLUSION

Free fatty acids and cholesterol are increasingly recognized as bioactive molecules affecting a wide range of tissues and biological processes. Studies aimed at understanding the involvement of obesity in the pathogenesis of osteoarthritis focus more and more on lipids as soluble factors increased in obesity. However, the experimental evidence both in preclinical models and humans is still scarce. Especially observational studies investigating the association of lipids with disease development or severity of disease in humans are lacking. Similarly, interventional and mechanistic studies in animal models especially regarding newly described fatty acid-derived lipid classes are upcoming, though still limited. Despite the limited availability of data regarding osteoarthritis, the pleiotropic biological actions of free fatty acids and cholesterol indicate them as promising candidates for future therapeutic interventions.

Acknowledgements

The authors would like to thank Eric van Spronsen for help with the art work.

Financial support and sponsorship

The authors' work is supported by the Dutch Arthritis Foundation (LLP24; A.I.-F., M.K.) and the Innovative Medicine Initiative Joint Undertaking funded project Be The Cure (115142–2; A.I.-F.).

Conflicts of interest

There are no conflicts of interest.

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What did we learn from 'omics' studies in osteoarthritis

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Purpose of review
'Omics' technologies developed for the massive analysis of the major biologically relevant molecules (genes, proteins, metabolites) have been applied to the study of osteoarthritis (OA) for more than a decade.

Recent findings
'Omics' studies have undoubtedly contributed to increase the knowledge on pathogenic processes related with OA and have provided hundreds to thousands of molecules that might have a putative biomarker utility for this disease.

Summary
This review describes the most recent 'omics' studies in OA research, their conclusions, and discuss those remaining challenges. Still many validation studies must be performed in large and well-characterized cohorts for the translation of the findings from 'omics' strategies to clinical applications. The development of tools for the intelligent integration of 'omics' data with clinical and imaging information is also mandatory to take full profit of the work that has been already performed.

Keywords
biomarkers, genomics, metabolomics, osteoarthritis, proteomics

INTRODUCTION

The 'omics' technologies were developed for the large-scale analysis of the major biologically relevant molecules: DNA, RNA, proteins, and metabolites (amino acids, lipids, etc.). Over the last decade, these technologies have been extensively applied for the study of osteoarthritis (OA) pathogenesis and for the discovery of novel molecules with marker usefulness for this disease. This review will describe the most recent 'omics' studies in OA research and their conclusions and discuss those remaining challenges.

RECENT ACHIEVEMENTS IN GENOMICS STUDIES

In genomics, recent technological advances expanded the amount of 'omic' data to higher levels, including genome sequencing, epigenetics, and transcriptomics. The aims of each of these molecular approaches are the understanding of the mechanisms underlying OA, and the identification of molecular markers to predict disease onset and progression (Table 1).

During the last decade, genome-wide association studies (GWAS) have been the preferred tool to study the genetics of late onset knee, hip, and hand OA [1–3]. Results derived from these studies reported that, as for many complex diseases, there is no single genetic variant responsible for OA. However, up to thousands of loci could be potentially associated, each with a small effect [4▪]. To date, this method of analysis identified 19 independent susceptibility loci for OA [4▪]. Some of them, such as rs1180992 at DOT1L gene, rs2862851 at TGFA, rs10471753 at PIK3R1, rs2236995 at SLBP,

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Curr Opin Rheumatol 2018, 30:114–120
DOI:10.1097/BOR.0000000000000460

KEY POINTS

- The high complex nature of OA has hindered the development of tools for the precise evaluation and therapeutic management of this disease.
- 'Omics' technologies have contributed to increase the knowledge on OA pathogenesis and have provided lists of molecules related with this disease.
- Further validation studies are still needed to translate the findings from 'omics' studies to clinical applications.
- A tool able to combine the molecular information generated by the 'omics' studies with imaging and clinical data would be highly valuable to facilitate precision medicine strategies in OA.

rs496547 at TREH, and rs10948172 at RUNX2, are also significantly associated with a decreased cartilage thickness in terms of clinical radiographic endophenotyping according to mJSW (minimal joint space width) [5]. The study of endophenotypes is a way to increase power in GWAS, enabling the detection of genes with potential functional importance that were not revealed in previous case-control studies because of disease heterogeneity. This is the case of a recent meta-analysis of clinically relevant endophenotypes in hip OA, in which Panoutsopoulou *et al.* [6▪▪] reported suggestive evidence for association of six variants located in novel genes such as LRCH1 or STT3B with increased joint space narrowing and the bone-remodeling response. A very recent study performed a GWAS of total hip replacements based on variants identified through whole-genome sequencing, concluding that two variants, a missense mutation in the COMP gene and a frameshift variant in the CHADL gene, were significantly associated with an increased risk of hip replacement [7].

Mitochondrial genetics has also been consolidated as a contributor to the risk of knee OA. Two recent meta-analyses showed a significant association of specific mtDNA variants with the rate of incidence and progression of OA in well-defined

Table 1. Genomics studies in OA disease from 2016

Type of study	Trait/phenotype	Findings	References
Review GWAS	OA susceptibility	Description of the most robust SNPs associated with OA with genome-wide significance	[4▪]
GWAS	mJSW	Six variants associated with decreased cartilage thickness	[5]
Meta-analysis	JSN and bone remodeling	Six variants associated with increased JSN and bone remodeling response	[6▪▪]
WGS/GWAS	Hip replacement	Two variants associated with increased risk of hip replacement	[7]
Genetic association	Incident knee OA	mtDNA haplogroup J associated with a decreased rate of incident knee OA	[9▪]
Genetic association	Rx knee OA progression	mtDNA cluster JT associated with a decreased risk of Rx knee OA progression	[8]
Genome-wide methylation in bone and cartilage	Stages of OA	Changes in subchondral bone precede the methylation changes in cartilage	[13▪▪]
DNA methylation	Epigenetic aging in cartilage	Premature epigenetic aging as a characteristic of OA cartilage	[15]
Gene expression array in BMSCs	Regenerative potential of BMSCs in advanced OA	690 intergroup differentially regulated genes between OA and healthy controls	[18]
RNA-seq in hMSCs	Differential expression patterns between OP and OA	Mechanisms stimulating hMSCs proliferation and mechanisms impairing their terminal differentiation as areas of potential interest for new therapeutic targets	[17]
Bioinformatic analysis of gene expression patterns of SM	OA progression	401 upregulated genes involved in inflammatory response and 196 downregulated genes involved in cell cycle	[19]
Gene expression between cartilage and synovium	OA susceptibility	There are different pathogenic mechanisms that are specific for the synovium in OA	[20]
Circulant miRNAs in serum	OA severity	miRNA let-7e as a potential predictor for severe knee or hip OA	[21]
Circulant miRNAs in SF	Stages of OA	Seven circulating miRNAs differentially expressed between late-stage OA and early-stage OA	[22]

BMSCs, bone marrow stromal cells; GWAS, genome-wide association studies; hMSCs, human mesenchimal stem cells; JSN, joint space narrow; miRNA, micro RNA; mJSW, minimum joint space width; mtDNA, mitochondrial DNA; OA, osteoarthritis; OP, osteoporosis; SF, synovial fluid; SM, synovial membrane; SNP, single-nucleotide polymorphism; WGS, whole-genome sequencing.

prospective cohorts such as the Cohort Hip and Cohort Knee (CHECK) and the Osteoarthritis Initiative (OAI). In these studies, haplogroup J and superhaplogroup JT associate with a decreased rate of incident knee OA at 8 years and a decreased rate of radiographic progression, respectively [8,9▪].

DNA methylation in OA has also been the focus of many studies during the last years. The first studies relied on the analysis of specific CpG sites in promoter regions of candidate genes involved in the OA process such as the matrix metalloproteinases [10]. However, the last works were based on genome-wide DNA methylation analyses in articular cartilage [11,12]. In the last year, a very interesting study analyzed DNA methylation changes in three regions of the subchondral bone of the tibial plateau to represent early, intermediate, and late stages of OA, and compare them with those on the site-matched cartilage. Zhang *et al*. [13▪▪] concluded that methylation changes in the subchondral bone could precede changes in the cartilage. All these studies show a high variable number of differentially reported sites. However, enrichment analyses of all of them indicate similar pathways, including embryonic morphogenesis, inflammation, and skeletal development [14]. Finally, according to OA methylation changes, Vidal-Bralo *et al*. [15] concluded that premature epigenetic aging is a characteristic of OA cartilage, being a component of the disease pathogenesis that reflects damage and vulnerability.

Several studies have used arrays to investigate gene expression changes in OA, mainly in articular cartilage [16]. However, over the last years, these transcriptomic assays were performed primarily to validate methylation analyses [11,17] and actually are being substituted by the most sensitive RNA-seq assay. Considering the proposed hypothesis that the regenerative potential of human mesenchymal stem cells (hMSCs) is altered in advanced-stage OA, some studies analyzed the methylation and/or expression profile of these cells. One of these works performed a large-scale gene expression profile of bone marrow stromal cells (BMSCs) from osteoarthritic cells compared with healthy, using a microarray from Affymetrix. This study revealed up to 690 intergroup differentially regulated genes between BMSCs from OA donors and healthy controls [18]. Another relevant work described a validation transcriptomic analysis by RNA-seq to compare the expression patterns of hMSCs from patients with fractures and OA. In this study, Del Real *et al*. [17] denote two areas of potential interest for discovering new therapeutic targets for bone mass disorders and bone regeneration: those related to the mechanisms stimulating MSCs proliferation and those impairing their terminal differentiation. Finally, the availability of data from the Gene Expression Omnibus (GEO) database permitted the bioinformatic analyses and meta-analyses of gene expression profiles. This is the case of one study of disease-related genes of synovial membrane associated with progression of OA, in which Dong *et al*. [19] identified 401 upregulated genes involved in the inflammatory response and 196 downregulated genes related with cell cycle processes. Another work identified a small overlap between the differentially expressed genes of the cartilage compared with those of the synovium. Park and Ji [20] suggest the existence of different pathogenic mechanisms that are specific of the synovium, although a much higher amount of differentially expressed genes were found in this tissue when comparing OA samples with healthy controls.

Finally, a relevant number of studies were performed most recently to discover differentially expressed micro-ribonucleic acids (miRNAs) in cartilage or bone between OA and controls. However, there is almost no overlap between those reported to be differentially expressed with statistical significance [14]. Analyses of circulating miRNAs in serum or synovial fluid, which may reflect altered tissue expression in OA and have thus a potential use as disease biomarkers, are of special interest. The first study on this area was conducted by Beyer *et al*. in a large prospective cohort consisting of 816 Caucasian individuals, and explored the association between serum levels of miRNAs and the development of severe OA. Beyer *et al*. [21]identified the miRNA let-7e as a potential predictor for severe knee or hip OA. A more recent study aimed to identify miRNAs in synovial fluid useful to differentiate between early- and late-stage knee OA, and led to the identification of a panel of seven circulating miRNAs [22].

CHARACTERIZATION OF OSTEOARTHRITIS RELATED-PROTEINS AND IDENTIFICATION OF PUTATIVE BIOMARKERS BY PROTEOMICS

After more than a decade of proteomics studies performed in OA, these approaches have contributed to a better understanding of disease pathogenesis and the identification of novel protein markers. The most recent descriptive studies in this field have been focused in elucidating the molecular composition of cartilage and the disease-dependent changes that occur in this tissue. This has been achieved by qualitative and quantitative shotgun analyses of the different layers and types (knee or hip) of OA and healthy tissues [23▪], and also by the evaluation of the response to IL-1α in cartilage using an in-vitro

Table 2. Circulating proteins with putative biomarker utility for OA found in the most recent proteomic studies

Protein	Biomarker utility	References
C3, ITIH1, S100A6	Knee OA diagnosis	[28▪]
PLTP, NRAM1/SLC11A1	Knee OA diagnosis	[29]
HPT, VWF	OA diagnosis	[30]
Glycated, oxidized and nitrated proteins, and amino acids	Early OA diagnosis	[31]
sCTXI, sHA, sNTXI, uC2C-HUSA, uCTXII, uNTXI, uCTXIα, uCTXIβ	Knee OA pain and structural worsening prediction[a]	[44▪▪]

C3, complement C3; HPT, haptoglobin; ITIH1, inter-alpha trypsin inhibitor heavy chain H1; NRAM1/SLC11A1, natural resistance-associated macrophage protein 1; OA, osteoarthritis; PLTP, phospholipid transfer protein; VWF, von Willebrand factor.
[a]These protein biomarkers were not found by proteomics, but have been recently described as predictors of case status in knee OA.

model of mouse tissue explants [24]. The analysis of chondrocyte secretomes has been reported as a valuable strategy to explore the processes related with extracellular matrix remodeling and the molecular mechanisms driven by the cell in response to different stimuli [25]. Accordingly, a recent proteomic study on secretomes reports the effects of nicotine on both OA and healthy chondrocytes treated with IL-1β, and suggests a negative effect of this drug on the joint [26]. Apart from these shotgun studies, the progression of matrix degradation in response to mechanical damage and cytokine treatment of human knee cartilage explants has been also evaluated using targeted proteomics [27]. Different protein domains of aggrecan, COMP neoepitopes, and collagen pro-peptides were measured throughout a 21-day culture period, being some of them potentially relevant as biomarkers for posttraumatic OA.

Biological fluids, such as synovial fluid, plasma, and serum, have been also extensively studied for the search of protein markers for OA (Table 2). In a discovery step, suspension bead-based protein arrays were used to screen serum samples from a cohort including OA and rheumatoid arthritis (RA) patients, and healthy controls. After linear regression analysis adjusting for sex, age, and body mass index (BMI) three proteins were significantly elevated in serum from OA patients compared with controls: C3, ITIH1, and S100A6. A panel consisting of these three proteins had an area under the curve of 0.82 for the classification of OA and control samples [28▪]. In an analogous study, a panel of seven proteins was found quantitatively different in sera from OA, RA, and healthy controls [29]. A targeted proteomic analysis was also performed on sera from OA patients and controls, in this case employing mass spectrometry [30]. The authors developed a method for the multiplexed monitoring of 14 biomarker candidates for OA, and verified the increased amount of Haptoglobin and von Willebrand Factor in OA patients.

Finally, protein modifications have also been recently explored using proteomic approaches. In one of this works, glycated, oxidized and nitrated proteins and amino acids were detected in synovial fluid and plasma of arthritic patients. Their combination with hydroxyproline and anti-CCP antibody status provided a plasma-based biochemical test of 0.92 sensitivity and 0.90 specificity for early-stage OA [31]. Using a very different strategy, N-glycosylation of proteins was analyzed by mass spectrometry imaging (MSI) in subchondral bone from knee OA patients [32]. The latter study demonstrates the usefulness of this novel technology to complement the proteomic data with valuable spatial information [33▪▪].

METABOLOMIC APPROACHES TO PROFILE OSTEOARTHRITIS-RELATED PROCESSES AND IDENTIFY NOVEL BIOMARKERS

Being the youngest of the 'omics' technologies, methods for the study of the metabolome have greatly evolved in the last years, also in the field of OA.

Two metabolomic approaches were recently carried out to gain insight into pathogenic processes characteristic of OA, such as subchondral bone sclerosis or osteophyte formation. In the first study, a metabolic profiling was carried out on subchondral bone from patients with primary OA [34]. Sixty-eight metabolites were identified to be significantly changed in the sclerotic tissue compared with the nonsclerotic. Metabolites such as taurine, hypotaurine, beta-alanine, L-carnitine, and glycerophospholipids were found to be related with this pathological process. In the work on osteophyte formation, Xu *et al.* [35] found metabolic variations between extracts of osteophyte cartilage tissues and uninvolved control cartilages, which are related with processes of collagen dissolution, destruction of boundary layers, and self-restoration. Phenylalanine metabolism was also highly correlated with osteophyte formation.

Regarding the identification of metabolites with putative biomarker usefulness for the disease,

Table 3. Metabolites with putative biomarker usefulness in OA, described in the most recent metabolomic studies

Metabolite	Type of sample	Biomarker utility	References
Malate, ethanolamine, squalene, glycerol, myristic acid, oleic acid, lanosterol, heptadecanoic acid and capric acid.	Synovial fluid	Diagnosis, progression	[36]
Glutamine, 1,5-anhidroglucitol, gluconic lactone, tyramine, threonine and 8-aminocaprilyc acid	Synovial fluid	Knee OA diagnosis	[37]
Polyunsaturated fatty acids, LOX, and COX pathway markers	Synovial fluid	Diagnosis	[38▪]
Arginine (low concentrations)	Plasma	Knee OA diagnosis	[39]
Branched-chain amino acids/histidine, lysoPCs/PCs	Plasma	Diagnosis, prognosis	[40]
Glycolate, hippurate, trigonelline	Urine	Progression predictors	[41▪]
Alanine, N,N-dimethylglycine, glycolate, hippurate, histidine, trigonelline	Urine	Progression markers	[41▪]

COX, ciclooxygenase; LOX, lipoxygenase; lysoPCs, lysophosphatidylcholines; OA, osteoarthritis; PCs, phosphatidylcholines.

metabolomic analyses have been performed recently in synovial fluid, plasma, and urine from OA patients. The conclusions from these studies are summarized in Table 3. In synovial fluid, 28 metabolites (including malate, ethanolamine, squalene, glycerol, myristic acid, oleic acid, lanosterol, heptadecanoic acid, and capric acid) were identified as critical metabolites for discriminating between early and late OA. These were robustly altered along the radiographic stage of knee OA [36]. In a similar study, six different metabolites (glutamine, 1,5-anhydroglucitol, gluconic lactone, tyramine, threonine, and 8-aminocaprylic acid) were strongly associated with knee OA. Gluconic lactone concentration was also significantly different between OA and RA [37]. Finally, a recent lipidomic analysis has been carried out, describing the identification of 37 lipids in the soluble fraction of SF from OA and RA patients. Among them are polyunsaturated fatty acids and their pro-inflammatory and pro-resolving lipoxygenase (LOX) and cyclooxygenase (COX) pathway markers. This work shows for the first time that resolution pathways are present in SF from OA patients [38▪].

In plasma, Zhang *et al.* [39] reported the identification of lower arginine concentrations in patients with knee OA compared with controls. They hypothesize that this is because of an over activity of arginine to ornithine pathway, which leads to an imbalance between cartilage repair and degradation. In another study from the same group, the branched-chain amino acids to histidine ratio was confirmed to be associated with advanced knee OA, and also the lysophosphatidylcholines (lysoPCs) to phosphatidylcholines (PCs) ratio. Subjects with this latter ratio at least 0.09 were 2.3 times more likely to undergo total knee replacement than those with the ratio less than 0.09 during a 10-year follow-up [40].

Finally, a very interesting study described metabolomic profiles in urine, distinguishing OA progressors (at least 0.7 mm decrease in JSW at 18 months) from nonprogressors ($\leq$0.35 mm decrease in JSW). Glycolate, hippurate, and trigonelline were among the important metabolites for discriminating these groups at baseline, whereas alanine, N,N-dimethylglycine, glycolate, hippurate, histidine, and trigonelline were among the metabolites that were important at 18 months. These findings support a role for metabolic factors in the progression of knee OA, and suggest that measurement of metabolites could be useful to predict progression [41▪]. Altogether, metabolomic studies have reported a number of molecules that play a role in the pathogenic process of OA and may be useful markers for disease progression studies.

REMAINING CHALLENGES

Given the great amount of information that the 'omics' approaches has provided to the investigation in OA, still there is a bottleneck in translating these findings to useful tools in clinical routines. Validation studies, capable to monitorize panels of biomarker candidates and qualify them for a clinical application, are still minority. In genomics, several meta-analyses have been performed for the systematic evaluation of the findings obtained in GWAS, and some validation studies have led to the definition of polymorphisms associated with OA susceptibility, severity, and rate of progression. Such type of analyses is yet almost absent in the field of proteomics and metabolomics, because of the higher complexity of the multiplexed analysis of metabolites and (even more) proteins. In this field, the ultimate advances in targeted proteomics and metabolomics technologies (such as mass spectrometry instrumentation and protein microarray platforms) are expected to facilitate their application in larger cohorts and under the frame of clinical trials.

To date, clinical data have been combined with the evaluation of genetic polymorphisms to predict primary knee OA progression [42], and also with imaging markers to generate prediction algorithms of structural progression [43]. Furthermore, a recent study evaluated the predictive validity of 18 protein biomarkers in serum and urine samples from the OAI cohort [44▪▪]. Considering the promising results obtained in these independent works, the next objective would be to develop combined tools (genes+proteins+metabolites+imaging) to identify patients with high risk of progression who will respond to a specific treatment. The integration of 'omics' information with clinical and imaging data is a highly promising strategy for the identification of phenotype profiles. The APPROACH project (Applied Public–Private Research enabling OsteoArthritis Clinical Headway, https://www.approach-project.eu/), currently ongoing, contributes to this integration by combining biomedical information (clinical, genomic, proteomic, metabolomic, X-ray and MRI) from knee OA patients and controls into a unified bioinformatics platform.

CONCLUSION

'Omics' technologies applied in the last decade for the study of OA have provided thousands of molecules related with this disease. Further validation of these findings will allow moving from single to multiplex biomarkers, defining the so-called molecular signatures related with a specific OA phenotype, or either those that could contribute to an increased diagnostic accuracy, disease progression studies, or to predict the response of each patient to a treatment. Leveraging multiomics technology to combine this information with the clinical data may much better define these biomarker profiles and further the goal of precision medicine strategies in OA.

Acknowledgements

We would like to thank all people from the Grupo de Investigación de Reumatología (GIR) for their daily work.

Financial support and sponsorship

This work was supported by grants from Fondo Investigación Sanitaria-Spain (grant number PI14/01707, PI14/01254, and PI16/02124) integrated in the National Plan for Scientific Program, Development and Technological Innovation 2013–2016 and funded by the ISCIII-General Subdirection of Assessment and Promotion of Research – European Regional Development Fund (FEDER) 'A way of making Europe'; CIBER(- grant number CB06/01/0040, RETIC-RIER-RD12/0009/0018, and PT13/0001). C. Ruiz-Romero and I. Rego-Pérez are supported by the Miguel Servet program from Fondo Investigación Sanitaria-Spain (grant number CPII15/00013 and CP12/03192).

Conflicts of interest

There are no conflicts of interest.

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Development and use of biochemical markers in osteoarthritis: current update

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Purpose of review
There is an increasing demand for noninvasive and descriptive biochemical markers (biomarkers) in osteoarthritis; for enabling early drug development (including translational research), evaluating clinical trial at an early stage and for subtyping. Purpose of the review is to review and comment on current availability of such biomarkers.

Recent findings
Many different biomarkers have been tested in the last 18 months. The main focus has been on testing whether the biomarkers, whether is reflect joint tissue turnover or inflammatory status, can differentiate osteoarthritis patients from healthy controls or whether the biomarkers are associated with progression. Less than a handful of studies, investigate the role of the biomarkers as response markers. Thus, there is still a great need for developing biomarkers that reflect disease activity and thereby can be used for treatment response or patient phenotyping.

Summary
Osteoarthritis is the most common form of joint disease. This presents the osteoarthritis research community and pharmaceutical companies developing disease-modifying osteoarthritis drugs (DMOADs) with great opportunities. There are different osteoarthritis subtypes, which complicates the traditional approaches for developing new treatments. If we can identify new markers that can distinguish different subtypes, this can greatly facilitate drug development from early discovery to late clinical development.

Keywords
biochemical marker, biomarker, cartilage, drug discovery, osteoarthritis, synovium

INTRODUCTION

Osteoarthritis is the most common form of joint disease that affects somewhere between 10 and 20% of the adult population and represents a major social economic burden [1]. Osteoarthritis is a highly heterogeneous disease characterized by several different subtypes and each is thought to have different risk factors and drivers. Although there is significant overlap between some of these phenotypes, there are still no clear tool for separation of these phenotypes [1,2]. This presents the osteoarthritis research community and pharmaceutical companies developing disease-modifying osteoarthritis drugs (DMOADs), challenges in developing new treatments. DMOAD have been trialed and failed, which may be attributed to a lack of clear phenotypes and insensitive markers of disease activity allowing tracking of treatment response. On the other hand, the diverse subtypes present new opportunities for developing targeted therapies for different forms of the disease. Thus, there is a clear medical need for objective, precise and accurate in-vitro diagnostic devices, and biomarkers, for early trial evaluation and decision-making, trial enrichment [3▪▪] and personalized healthcare in osteoarthritis [4▪▪].

Blood-based or urine-based biochemical markers (biomarkers) are one class of markers, which may be used to facilitate drug development and guide diagnosis or profiling of patients. They are noninvasive (well tolerated) and objective and can, therefore, be used for frequent monitoring of disease activity. Different biomarkers may be used for

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Curr Opin Rheumatol 2018, 30:121–128
DOI:10.1097/BOR.0000000000000467

KEY POINTS

There are many interesting biomarkers being tested in the field, but there still a lot focused work needed to develop:

- Translational and disease activity biomarkers, which allow better characterization of a drug in preclinical development.
- Early identification of efficacy of intervention; go/no-go decision-making already in phase 1b–2a studies, which normally do not include efficacy measures.
- Phase-II and Phase-III trial enrichment; reduction in study size and length to allow more efficient and less costly trials.
- Identification of patients who are fast progressors and super-responders to given treatment and as such in greatest need of treatment.
- Easy accessible monitoring devices – point of care, postmarketing patient care and personalized medicine.

different purposes through the drug-development phases (Fig. 1). The key message, from the figure, is that there a need for many types of biomarker fulfilling different functions; from pharmacodynamic markers used in humans or animals to companion diagnostics utilized by physician and patients. One single biomarker cannot accomplish all. Consequently, there is a strong development need for sensitive biomarkers that can be used as predictive and surrogate markers for clinical endpoints for to enable earlier, strategic, more rational and cost saving go/no-go decisions.

BIOMARKERS OF DISEASE ACTIVITY

Osteoarthritis is a disease affecting the entire joint, including tissues cartilage, synovium and subchondral bone. In addition, data have also pointed toward that there may be both inflammatory and metabolic components to the disease. In order to use the biochemical markers to categorize patients and providing the optimal treatment or care, it is necessary to understand what the biomarkers reflect. In essences, most biomarkers are markers of disease activity. Some reflect what is going on in the tissue, such as circulating matrix metalloproteinases (e.g. MMP-3) or tissue metabolites (e.g. CTX-II). Others reflect inflammation (e.g. the cytokine IL-6), regulation (e.g. hormone such as estrogen) or metabolic status (e.g. glucose levels). There are more and more data coming out that tissue metabolite, for example, can act as pro-inflammatory regulators, thus there may not be a clear separation between the marker classes. Whether the markers reflect inflammation or tissue metabolism, these provide information on disease activity; some are general markers common

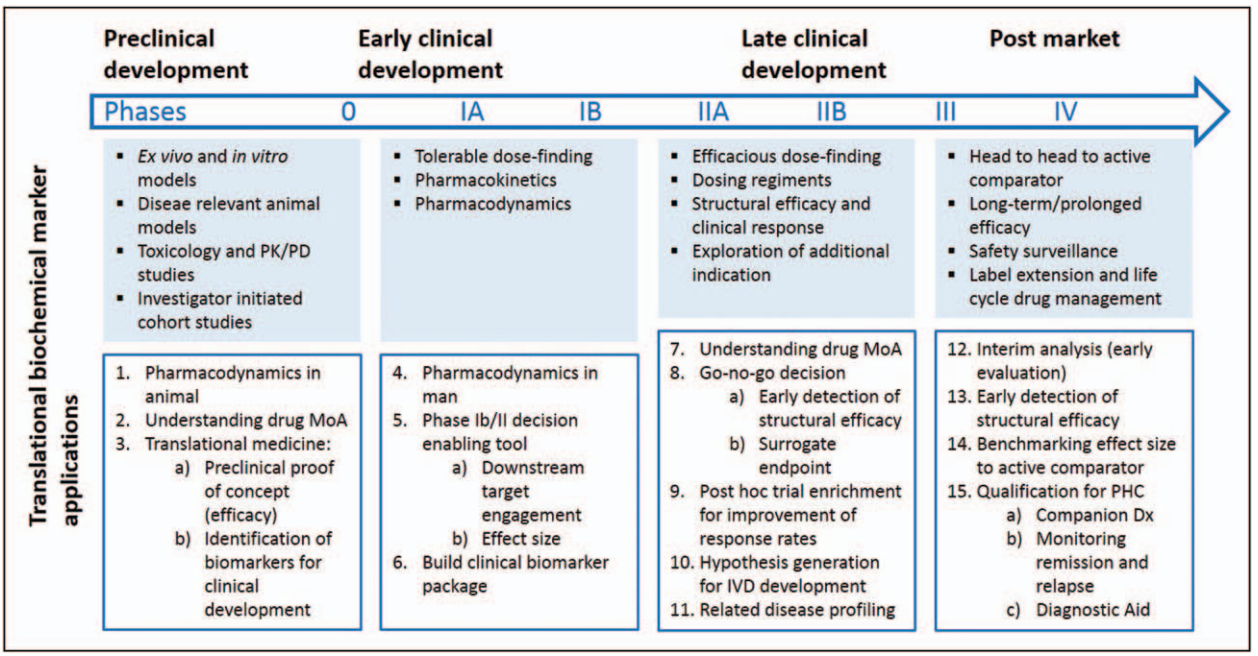

FIGURE 1. There is a need for biomarkers at different phases of drug development. Usage of biomarker in clinical drug development, from preclinical development to late clinical development, and postmarket applications. The blue boxes indicate the major objectives where biomarkers may be used as drug-development tools (DDTs). The open boxes provide specific suggestions to applications.

for all subtype of disease, whereas others are related to specific disease subtypes. One example is CTX-II, which reflect articular and calcified cartilage degradation or calcified cartilage degradation alone, where data suggests that there is a link to radiographic progression [5]. This may indicate that CTX-II is a disease activity biomarker of cartilage–bone and ultimately point toward it being a marker a cartilage–bone-driven phenotypes. Of cause, it is not as simple as this, however, it illustrates where the osteoarthritis biomarker field is going.

There are several recent and older, but still valid, reviews publicly available on different biomarkers tested and used in osteoarthritis over the last decades [6–8,9▪,10,11]. Thus, the objective of current review is to provide an overview of recent data published in the field.

BIOMARKERS OF TISSUE DISEASE ACTIVITY

Biomarkers of cartilage turnover

The healthy articular cartilage is an avascular tissue with a slow remodeling rate. The single predominant cell type in cartilage is the chondrocyte. They are sparsely distributed in the cartilage and are critical in the maintenance of the ECM because of the lack of vascularization in the cartilage. The extracellular matrix composition of cartilage consists largely of proteoglycans and collagen type II, but types IX–XI collagens are also present [12]. The main role of the collagens is to act as the structural framework needed to keep aggrecan in place. The early stages of osteoarthritis development include cartilage deterioration at the superficial zone and general deterioration of the organized extracellular matrix (ECM) structure. As the disease progresses, the chondrocytes adapt a proliferative phenotype and become hypertrophic. Hypertrophy leads to increased protease expression, including matrix metalloproteinases (MMPs) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) and is associated with increased tissue turnover.

The cleavage products from the proteolytic burden associated with osteoarthritis have been identified as important biomarker targets that can be used to describe various aspects of the disease. The MMP-derived type-II collagen fragment CTX-II, have been measured in a long range of clinical studies. It has been established as a marker of cartilage degradation, and has been shown to correlate to the Kellgren Lawrence grade in osteoarthritis patients [13]. The MMP generated type-II collagen fragment C2M have been shown to be higher in patients with osteoarthritis compared with healthy controls [14]. With the development of inhibitors targeting the proteolytic activity of osteoarthritis-associated enzymatic process in aggrecan degradation, attention have also been given to cleavage fragments of the ADAMTS-4 and ADAMTS-5, such as ARGS and TEGE [15]. Table 1 provides an overview of recent publications, testing markers of cartilage turnover in osteoarthritis. What is worth noting is that the majority of the markers on the list are markers that have been well characterized. This may indicate that it has been difficult to develop and test markers reflecting cartilage turnover.

The meniscus is another cartilaginous tissue. It is composed of a medial and lateral component situated in between the tibial and femoral condyles, where it serves a vital role in weight-bearing and load transmission as well as in lubrication and nutrition of the articular cartilage [22,23]. The meniscus is a complex tissue composed of cells, an extensive extracellular meshwork, and in contrast to articular cartilage, it is somewhat vascularized and innervated in the outermost layers [24]. The meniscus, like cartilage, is a highly hydrated tissue with about 70% water. The remaining part consists of organic material, mainly cells and ECM. The meniscus is separated into two parts, which differ slightly in composition. The outer parts are vascularized structures consisting mainly of collagen type I (80%), and other collagens, types II– IV, VI, and XVIII. The less vascularized zones, deeper in the meniscus, are richer in collagen type II (60%) and less in collagen type I (40%). In addition to collagen, another fibrillar component is elastin, but at present, its physiological role is not clear. The other large component of the meniscus is proteoglycans. The main component is aggrecan, but also biglycan and decorin are present, and serve to absorb water. The glycoproteins, fibronectin, thrombospondin and collagen type VI, serve as adhesion molecules bridging the link between the ECM and resident cells [22]. Thus, the meniscus may be a substantial source of turnover markers.

Bone resorption and formation

Bone is a dynamic tissue, which is constantly remodeled in response to mechanical load, metabolic changes and microdamage. In healthy individuals, bone remodeling is a tightly balanced process where bone resorption performed by the osteoclasts removes old or damaged bone, which is then replaced by adequate amounts of new bone by the bone-forming osteoblasts. The relationship between bone turnover and osteoarthritis has been clearly established in the osteoarthritis field, and has

Table 1. Recent data on cartilage turnover biomarkers, which show association with osteoarthritis diagnosis, prognosis and treatment response

Biomarker	Description	Recent data	Test type
ARGS	ADAMTS cleavage neoepitope of aggrecan	ARGS was measured in the synovial fluid of 39 anterior cruciate ligament injury patients and 32 controls with normal knees. There were a significant association with pain and a correlation to improvement in pain over 10 weeks [16]	Diagnostic; burden of disease
COMP	Total cartilage oligomeric protein	COMP and COMPneo were measured in synovial fluid of osteoarthritis, rheumatoid arthritis, reactive arthritis and acute joint trauma patients [17]. It was found that COMP was higher in osteoarthritis patients and the COMPneo was higher in the acute joint trauma patients; serum COMP and urinary CTX-II were measured in 1335 participants of the Rotterdam study 2 and high levels were found to be positively associated with osteoarthritis radiographic progression with an odd ratio of 1.2–1.3 [18]. The markers were likewise associated with incidence osteoarthritis; serum COMP was measured in 593 women with no radiographic knee osteoarthritis at baseline. The highest quartile of COMP was associated with increased 20-year risk of developing osteoarthritis with an odds ratio of 2 [19]	Burden of disease; prognosis
COMPneo	Neoepitope of cartilage oligomeric protein	See under COMP	Burden of disease
PIIANP	Propeptide of type II collagen, isotype A	Serum PIIANP was predictive of progression the OAI- FNIH case–control study ($N = 194/406$) with an odds ratio of 0.8–0.9, whenever doing time-integrated concentration analysis (baseline to 24 months) [5]	Prognosis
uC2C	Urinary collagenase-mediated degradation fragment of type-II collagen	uC2C was measured in 253 patients with knee pain from a population-based cohort. There was a statistically significant difference between patients with no sign of cartilage damage, early signs of osteoarthritis and radiographic osteoarthritis, where the highest level was found in the radiographic osteoarthritis group [20]. High level of uC2C could predict progression of osteoarthritis with an odds ratio of 1.8; similarly, uC2C was predictive of progression the OAI-FNIH case–control study ($N = 194/406$) with an odds ratio of 1.3–1.5, whenever doing time-integrated concentration analysis (baseline to 24 months) [5]	Diagnosis; prognosis
uCTX-II	Neo-epitope fragment of type-II collagen teleopeptide	In a placebo-controlled trial including 49 patients with anterior cruciate ligament injury, uCTX-II was shown to be significantly higher in the placebo compared with groups treated with corticosteroid [21]; uCTX-II was at baseline predictive of progression the OAI-FNIH case–control study ($N = 194/406$) with an odds ratio of 1.3 [5]; see also under COMP	Prognosis; efficacy

Test type suggests what type of implication for the biomarker, the study provide according to the BIPED (burden of disease, investigative, prognostic, efficacy of intervention, and diagnostic) criteria. OAI-FNIH, Osteoarthritis Initiative-Foundation for the National Institute of Health.

received substantial interest in recent years [25,26,27▪,28]. This relationship seems to be largely dependent on stage and origin of disease. The bone effects appear to be largely isolated to changes in the subchondral compartment of the joint [29,30], although the development of osteophytes have also been shown to be important for the development of pain in later disease stages, and is a hallmark of osteoarthritis disease [31]. Changes in the subchondral region are associated with increased vascularization, significant bone marrow changes, bone marrow lesions, and increased microfractures all leading to substantial increases in bone remodeling and tissue turnover [32].

Several biomarkers exist for the measurement of bone matrix tissue turnover. C-terminal telopeptide of collagen I (CTX-I) measures the degradation of collagen type I by the cysteine protease cathepsin K secreted by osteoclasts during bone resorption [33]. CTX-I, also exists in an alpha-isomerized form associated with the turnover of young bone [34,35]. In a longitudinal study, alpha CTX was associated with increased subchondral bone turnover measured by bone scintigraphy and associated with osteoarthritis progression based on osteophyte score and joint space narrowing (JSN), in patients with symptomatic and radiographic osteoarthritis [36]. Table 2 provides an overview of recent publications, testing markers of bone resorption and formation in osteoarthritis. As with the cartilage markers, there seem to be an overrepresentation of 'old-timers'.

Synovial inflammation and fibrosis

The synovial membrane is a thin cellular structure, which encapsulates the joint cavity from the external endothelial cell structures. The membrane consists of the intimae and the subintimae layer, which are distinctly different [41]. The cells in the intimae layer are embedded in in a meshwork of structural fibrils consisting of collagen types I–VI. The subintimae layer largely consisting of type-I collagen, is vascularized, but only contains few cells. These cells are mainly synovial fibroblasts and infiltrating cells. In inflammatory conditions, the synovium is infiltrated by immune cells, which induce fibrosis and neovascularization, also known as synovitis. This inflammation is accompanied by cellular changes in the form of cell hyperplasia of the membrane-lining cells [42]. The inflammatory state causes the synovial macrophages to produce increased amounts of pro-inflammatory cytokines, which in turn induced a pro-inflammatory state in the synovial fibroblasts resulting in increased protease and pro-inflammatory cytokine production [43]. The increased inflammation and the accompanying

Table 2. Recent data on bone and formation biomarkers, which show association with osteoarthritis diagnosis, prognosis and treatment response

Biomarker	Description	Recent data	Test type
DKK-1	Dickkopf-1	DKK-1 was shown be lower and OPG was higher in knee osteoarthritis patients as compared with healthy individuals ($N=49/101$) [37]. The markers were somewhat predictable for radiographic severity	Diagnosis; burden of disease
Gremlin-1	A bone morphogenetic protein (BMP) antagonist	Serum levels of gremlin-1 were measured in 212 osteoarthritis patients and 125 healthy controls; the levels were higher in osteoarthritis patients and synovial fluid was associated with severity of osteoarthritis [38]	Diagnosis; burden of disease
OPG	Osteoprotegerin	See under DKK-1	Diagnosis; burden of disease
OPN	Osteopontin	Plasma levels of OPN was measured in 21 patients with severe idiopathic hip osteoarthritis. Patients with Kellgren Lawrence score of 4 had significantly higher levels of OPN than those with a score of 3 [39]	Burden of disease
sCTX-I	Neo-epitope fragment of type-I collagen cross-linked teleopeptide	Serum CTX-I was predictive of progression the OAI-FNIH case–control study ($N=194/406$) with an odds ratio of 1.3, whenever doing time-integrated concentration analysis (baseline to 24 months) [5]	Prognosis
sNTX-I	Neo-epitope fragment of type-I collagen cross-linked teleopeptide	Serum NTX-I was predictive of progression the OAI-FNIH case–control study ($N=194/406$) with an odds ratio of 1.3, whenever doing time-integrated concentration analysis (baseline to 24 months) [5]	Prognosis
TRAPcP5b	Tartrate-resistant acid phosphatase; 5b	Serum TRAcP5b activity was associated with baseline pain and pain change in 129 patients from the POP cohort [40].	Burden of disease
uCTX-Ialpha	Neo-epitope fragment of type-I collagen cross-linked teleopeptide	Urine CTX-II was at baseline predictive of progression the OAI-FNIH case–control study ($N=194/406$) with an odds ratio of 1.2 [5]	Prognosis
uCTX-Ibeta	Neo-epitope fragment of type-I collagen teleopeptide	Urine CTX-I was predictive of progression the OAI-FNIH case–control study ($N=194/406$) with an odds ratio of 1.3, whenever doing time-integrated concentration analysis (baseline to 24 months) [5]	Prognosis

Test type suggests what type of implication for the biomarker the study provide according to the BIPED (burden of disease, investigative, prognostic, efficacy of intervention, and diagnostic) criteria. OAI-FNIH, Osteoarthritis Initiative-Foundation for the National Institute of Health; OPN, osteopontin; POP, prediction of OA progression.

proteolytic burden results in an increased turnover of the extracellular matrix consisting largely of types I and III collagen, and the release of ECM fragments, which can be measured in both blood and synovial fluid [44,45]. Table 3 provides an overview of recent publications, testing markers of synovial turnover and inflammation in osteoarthritis.

Inflammatory and metabolic markers

There is an array of different inflammatory and metabolic cytokines, myokines, hormones and metabolite involved in the development, homeostasis and progression of osteoarthritis. Tons of these have been tested as markers during the last decades both as single biomarkers and as panel of markers. The topic of these classes of biomarkers are too substantial for this review, however, Table 4 summarizes recent studies, testing some of these markers.

CONCLUSION

Looking at the overview tables presented here, it is striking that most of the biomarkers are investigated

Table 3. Recent data on synovial inflammation and fibrosis biomarkers, which show association with osteoarthritis diagnosis, prognosis and treatment response

Biomarker	Description	Recent data	Test type
C1M	Neo-epitope fragment of type-I collagen generated by MMP. Blood-based marker, measured by ELISA	Changes in serum C1M, C3M and CRPM levels were found to be positive, associated with change in weight in the IDEA study, including obese, elderly symptomatic osteoarthritis patients ($N=429$) [46]. The markers were associated with IL6, indicating a link between change in inflammatory status and synovial tissue markers	Efficacy
C3M	Neo-epitope fragment of type III collagen generated by MMP. Blood-based marker, measured by ELISA	See under C1M	Efficacy
CRPM	Metabolite of C-reactive protein generated by MMP. Blood-based marker, measured by ELISA	Serum CRPM and full-size CRP were measured in 1335 participants of the Rotterdam study 2 and high levels were found to be positively associated with osteoarthritis radiographic progression with an odd ratio of 1.3 for both [18]; see also under C1M	Diagnosis; prognosis

Test type suggests what type of implication for the biomarker, the study provide according to the BIPED (burden of disease, investigative, prognostic, efficacy of intervention, and diagnostic) criteria.

Table 4. Recent data on inflammatory and metabolic biomarkers, which show association with osteoarthritis diagnosis, prognosis and treatment response

Biomarker	Description	Recent data	Test type
Adipsin	Adipokine, aka complement factor D	Serum levels of adipsin and leptin were measured in 138 knee-osteoarthritis patients and were found to be associated with cartilage volume loss in lateral compartment and femur with an odds ratio of 2.9, and the medial compartment with an odds ratio of 2.9, respectively [47]	Burden of disease
α-MSH	α-Melanocyte-stimulating hormone	α-MSH was found, whenever measured in synovial fluid from 66 posttraumatic ankle osteoarthritis patients [48], to be inversely correlated with radiographic severity and cartilage degradation markers such as CTX-II	Burden of disease
CXCL12	CXC chemokine ligand-12	Plasma and synovial fluid of 244 patients with knee osteoarthritis and 244 age-matched and sex-matched healthy controls were assessed for CXCL12. Plasma CXCL12 levels were higher in osteoarthritis patients as compared and there was a positive correlation between levels of CXCL12 and Kellgren Lawrence grade [49]	Diagnosis; burden of disease
HIF-1α	Hypoxia-inducible factor 1α, transcriptional regulator	Compared with healthy controls, osteoarthritis patients ($N=36$) exhibited an increased HIF-1α concentration in synovial fluid. Furthermore, analysis showed that synovial-fluid HIF-1α levels were significantly correlated with the severity of disease [50]	Diagnosis; burden of disease
IL-1β	Interleukin 1 beta, pro-inflammatory cytokine	IL-1beta, TNFalpha, IL-8, IL-6 and TGFbeta were elevated in osteoarthritis patients compared with healthy age-matched controls, which was accompanied by altered levels of stress factors such as cortisol and the extracellular 72 kDa heat-shock protein [51].	Diagnosis
IL-21	Interleukin 21, cytokine	Serum IL-21 levels in osteoarthritis patients were significantly higher than those in healthy controls ($N=40/13$) and there was positive correlation with radiographic severity [52]	Diagnosis; burden of disease
IL-6	Interleukin 6, inflammatory cytokine and anti-inflammatory myokine	Plasma IL-6 and CRP levels were measure in 44 overweight and adult participants from the IDEA study, at baseline and at the 18-month follow-up. IL6 levels were found to decrease in the nonprogressors, whereas the level remained constant in the progressors [53]; see also under IL-1beta	Diagnosis; Prognosis
IL-8	Interleukin 6, aka CXCL8, chemokine	See under IL-1beta	Diagnosis
Irisin	Myokine, Cleavage product of Fibronectin type III domain-containing protein 5	Irisin and CRP were measured in serum samples of 215 patients with knee osteoarthritis and in healthy controls. Irisin was found to lower in osteoarthritis patients whereas CRP was higher. Irisin levels in serum and SF of knee osteoarthritis patients were negatively correlated with disease severity evaluated by Kellgren Lawrence grading criteria [54]	Diagnosis; Burden of disease
Leptin	Satiety hormone	See under Adipsin	Burden of disease
oxLDL	Oxidized low-density lipoprotein	oxLDL and PON1 were measured in 203 osteoarthritis patients and 194 controls. oxLDL was found to higher, whereas PON1 was significant lower in osteoarthritis patients [55]. There was a positive correlation between radiographic severity and oxLDL.	Diagnosis; burden of disease
Resistin	Adipocytokine, hormone	Resistin was measured in serum and synovial fluid of 74 knee osteoarthritis patients and 79 healthy controls. Synovial fluid levels were associated with WOMAC pain scores and radiographic osteoarthritis [56]	Diagnosis; Burden of disease
TGF-β1	Transforming growth factor beta, cytokine, growth factor	See under IL-1beta	Diagnosis
TNF-α	Tumor necrosis factor alpha, cytokine	See under IL-1beta	Diagnosis
UA	Uric acid	Uric acid was measured in 88 serum samples from patients with knee osteoarthritis and found to be independently correlated to joint severity and somewhat predictive of progression (AUC = 0.63). There was a correlation between serum and synovial fluid levels of UA [57]	Burden of disease

Test type suggests what type of implication for the biomarker the study provide according to the BIPED (burden of disease, investigative, prognostic, efficacy of intervention, and diagnostic) criteria. AUC, area under the curve; CRP, C-reactive protein.

as either diagnostic markers that can differentiate osteoarthritis patients from healthy controls or prognostic markers, which can predict progression of disease. However, is that actually fulfilling a medical need? Indeed, these types of investigations are needed to characterize and generate understanding of the individual markers. Nevertheless, it does not necessarily facilitate drug development or result in new diagnostic concepts. There is gap between biomarker research and real-life application. We believe that biomarker development need to be much more tightly bound to drug development, as drug development will help define the need for biomarkers for patient subgrouping and profiling (i.e. providing new platform for diagnosis and precision medicine), and for patient progression and response to treatment (i.e. prognosis and efficacy). Without this, biomarkers will have no change on earth.

Acknowledgements

We would like to acknowledge Dr Morten A. Karsdal and our research groups at Nordic Bioscience and University of Surrey for continuous scientific support.

Financial support and sponsorship

A.M., C.S.T. and A.C.B.J. are members of the D-BOARD Consortium funded by European Commission Framework 7 programme (EU FP7; HEALTH.2012.2.4.5-2, project number 305815, Novel Diagnostics and Biomarkers for Early Identification of Chronic Inflammatory Joint Diseases). A.M. is co-ordinator of the D-BOARD Consortium and member of the Arthritis Research UK Centre for Sport, Exercise, and Osteoarthritis, funded by Arthritis Research UK (Grant Reference: 20194). A.M. has received funding from the Deanship of Scientific Research (DSR), King Abdulaziz University (Grant No 1-141/1434 HiCi). The authors are also members of the Applied Public-Private Research enabling OsteoArthritis Clinical Headway (APPROACH) Consortium, a 5-year project funded by the European Commission's Innovative Medicines Initiative (IMI). APPROACH is a public-private partnership directed towards osteoarthritis biomarker development through the establishment of a heavily phenotyped and comprehensively analysed longitudinal cohort. The research leading to these results has received partial support from the Innovative Medicines Initiative (IMI) Joint Undertaking under grant agreement no. 115770, resources of which are composed of financial contribution from the European Union's Seventh Framework programme (FP7/2007-2013) and EFPIA companies' kind contribution.

Conflicts of interest

A.C.B.J. and C.S.T. are employees of Nordic Bioscience, which is a privately owned biotechnology company involved in the development and testing of biomarkers for inflammatory and fibrotic diseases. A.C.B.J. holds shares in Nordic Bioscience. A.M. declares that he has served as a Scientific Advisory Board Member for AbbVie.

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Extracellular vesicles in cartilage homeostasis and osteoarthritis

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Purpose of review
Extracellular vesicles carry bioactive molecules that can be transferred between cells and tissues. The purpose of this review is to describe how extracellular vesicles regulate functions of cells in cartilage and other joint tissues. The potential application of extracellular vesicles in the treatment of osteoarthritis and as biomarkers will also be discussed.

Recent findings
Extracellular vesicles are found in synovial fluid, in articular cartilage and in the supernatants of synoviocytes and chondrocytes. Extracellular vesicles in cartilage have been proposed to be involved in cross talk between cells in joint tissues and to affect extracellular matrix turnover and inflammation. Extracellular vesicles from arthritic joints can promote abnormal gene expression and changes in cartilage extracellular matrix, including abnormal mineralization. Promising results were obtained in the therapeutic application of mesenchymal stem cell-derived extracellular vesicles for cartilage repair and experimental osteoarthritis.

Summary
Extracellular vesicles have emerged as vehicles for the exchange of bioactive signaling molecules within cartilage and between joint tissues to promote joint homeostasis and arthritis pathogenesis. As the molecular content of extracellular vesicles can be customized, they offer utility in therapeutic applications.

Keywords
cell–cell communication, chondrocytes, exosomes, extracellular vesicles, osteoarthritis, synoviocytes

INTRODUCTION

Osteoarthritis represents the most common musculoskeletal disorder [1]. It is a complex and multifaceted disease, characterized by the degradation of articular cartilage, subchondral bone remodeling, joint inflammation and changes in meniscus and ligaments [2]. Various risk factors for osteoarthritis have been identified and these include aging, joint injury, excessive chronic mechanical stress, genetic factors and metabolic disorders [3]. Although several pathogenesis pathways have been characterized [4], current knowledge is incomplete and has not led to effective approaches for prevention or treatment. These limitations can be overcome by advances in the understanding of molecular mechanisms that are involved in the maintenance of joint tissues which involves communication of cells within the different joint tissues. Cells are able to communicate with neighboring or distant cells through cytokines and hormones. Recently, extracellular vesicles that are released from cells have attracted attention as novel a mechanism of cell–cell communication. Extracellular vesicles transfer bioactive molecules to recipient cells to modulate their activity [5]. In this review, we focus on the role of extracellular vesicles in cell–cell communication within and among joint tissues during homeostasis and osteoarthritis pathogenesis and address the potential therapeutic application of extracellular vesicles.

EXTRACELLULAR VESICLES

Extracellular vesicles had previously been regarded as inert cellular debris, which was generated as a

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Curr Opin Rheumatol 2018, 30:129–135
DOI:10.1097/BOR.0000000000000454

KEY POINTS

- Extracellular vesicles are released from chondrocytes through various mechanisms, including autophagy and apoptosis.
- Extracellular vesicles from chondrocytes can play a role in abnormal articular cartilage mineralization.
- Extracellular vesicles from MSCs can be enriched for certain bioactive molecules, such as miRNAs and used for tissue repair and in the treatment of osteoarthritis.

consequence of cell damage or the result of dynamic plasma membrane turnover. However, recently, cell–cell communication via extracellular vesicles has become the center of attention in research of diseases and tissue repair. Extracellular vesicles contain bioactive molecules, including proteins, mRNAs, microRNAs, lipids and DNA [6]. Extracellular vesicles have been classified depending on size and biogenesis pathway. Exosomes are small extracellular vesicles (30–150 nm in diameter) that are generated in multivesicular endosome/multivesicular bodies and are released when these compartments fuse with the plasma membrane. Microvesicles/microparticles (50–1000 nm in diameter) are released by budding from the surface of the plasma membrane [7]. Exosome biogenesis is a very tightly regulated process governed by multiple signaling molecules, and begins with receptor activation that is unique to each cell type [8]. Detailed and conclusive characterization of the various types of extracellular vesicles has not yet been accomplished. The International Society for Extracellular Vesicles provided a minimal set of biochemical, biophysical and functional standards [9]. Size alone cannot distinguish exosomes from microvesicles [10]. Furthermore, some proteins previously used as exosome markers, such as major histocompatibility complex class II or class I molecules, heat-shock proteins, flotillins or actin, are present in all types of extracellular vesicles, and thus cannot be considered as either exosome or even extracellular vesicle-specific markers [11]. New specific markers of medium and large size extracellular vesicles (e.g. actinins), of endosome-derived exosomes (coexpressing three tetraspanins CD9/CD63/CD81 and including TSG101 and syntenin-1), and of nonendosomal extracellular vesicles (some integrins) have been proposed [7,11]. There remains a continuing need to better understand the molecular mechanisms of the biogenesis and release of extracellular vesicles and to discover better markers for the various types. The released extracellular vesicles have surface receptors/ligands of their cell source and have potential to interact with specific target cells [6]. Extracellular vesicles directly stimulate target cells by receptor-mediated interactions or transfer of the enclosed bioactive molecules into the recipient cell [8].

EXTRACELLULAR VESICLES FROM CARTILAGE AND CHONDROCYTES

Extracellular vesicles have long been known to be present in the pericellular matrix of articular cartilage and growth plate cartilage [12–17]. Various terms have been used to describe them, including matrix vesicles (MaVs), articular cartilage-derived extracellular vesicles (ACEVs) or apoptotic bodies but there is no conclusive distinction (Table 1) [9]. Originally, MaVs were described in growth plate as derived from budding or disintegrating cells that are associated with hydroxyapatite deposition. Alkaline phosphatase activity is abundant in MaVs and is used as a marker for their identification [13]. MaVs also contain pyrophosphate-generating nucleoside triphosphate (NTP) pyrophosphohydrolase activities. MaVs can be isolated from collagenase-digested articular cartilage and separated from chondrocytes by differential centrifugation and used for

Table 1. General characteristic of articular cartilage-derived extracellular vesicles

	Exosome	Matrix vesicle/microvesicle	Apoptotic body
Origin	Endocytic pathway	Budding off/fusion from the Plasma membrane	Plasma membrane
	Autophagic pathway	Autophagic pathway	In apoptotic cell
[a]Size	30–150 nm	100–1000 nm	100 nm <
Marker	CD9, CD63, CD81, Flotillin-1, Alix, TSG101, LC3		
Content	mRNA, noncoding-RNA (microRNA), protein, nuclear fractions		
	Lipid, DNA, organelles		
Isolation method	Differential centrifugation/density gradient centrifugation		
	Commercial kit		

ACEV, articular cartilage-derived extracellular vesicle.
[a]Size alone cannot distinguish exosomes from EVs [9].

functional, biochemical and ultrastructural studies [18]. Isolated MaV can incorporate calcium, hydrolyze adenosine triphosphate or other nucleotide triphosphates, and facilitate precipitation of hydroxylapatite [19]. Although MaVs from different sources are heterogeneous [20], they are similar with respect to the capacity to mineralize matrix [12,17]. ACEVs have been shown to have a physiological function in endochondral bone development and pathologic role in calcium crystal deposition in articular cartilage [21▪▪]. The majority of the proteome was shared by extracellular vesicles isolated from normal and osteoarthritis cartilage, but immunoglobulins and complement components were present only in osteoarthritis ACEV which also contained lower levels of matrix proteoglycans [22]. Importantly, the ACEV proteome shares fewer similarities with exosomal proteomes. The heterotrimeric G proteins, HSP70 and 90 and members of the tetraspanin family such as CD9, CD63 and CD81 that are particularly characteristic of exosomes were not seen in ACEV [22]. CD9, CD63 and CD81 were previously considered to be specific markers for exosomes; however, in recent proteomics comparison, these proteins were observed in all extracellular vesicles including microvesicle and apoptotic bodies [11,23].

RNAs are also packaged in extracellular vesicles and are transferable genetic material from tissue to tissue and from human to human [5]. RNAs are protected from degradation by the lipid membrane of the extracellular vesicles. Coding and noncoding small RNAs in extracellular vesicles were in proportions that differed from parent cells with an enrichment of specific miRNAs suggesting that miRNAs are selectively packaged into extracellular vesicles. For example, small RNAs such as miRNAs were enriched in extracellular vesicles isolated from cultures of costochondral growth zone chondrocytes, whereas large RNAs such as 18S and 28S rRNA were not detected [24▪]. Extracellular vesicles from normal articular cartilage contain full-length mRNAs for factor XIIIA, type II transglutaminase, collagen II, aggrecan, ANKH inorganic pyrophosphate transport regulator and glyceraldehyde-3-phosphate dehydrogenase. When transferred to chondrocytes, ACEV-derived RNA was internalized. This was associated with changes in the expression of alkaline phosphatase and osteopontin [25].

The mechanisms of ACEV formation include apoptosis which is increased in osteoarthritis-affected cartilage [26]. Chondrocyte-derived apoptotic bodies contain alkaline phosphatase and NTP pyrophosphohydrolase activities, and can precipitate calcium [27]. A role of apoptosis in generating this type of ACEV has been demonstrated in

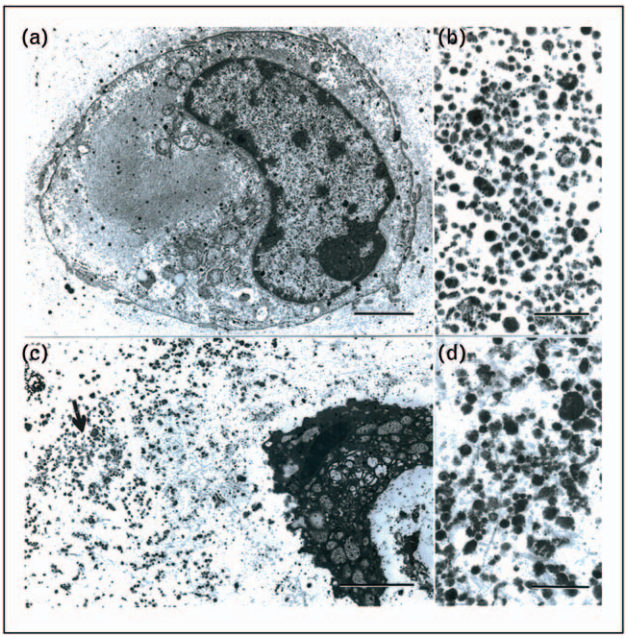

FIGURE 1. Electron microscopy of apoptotic chondrocytes, apoptotic bodies and matrix vesicles. (a) Electron micrograph of a chondrocyte from normal articular cartilage. Bar represents 2 μM. (b) Isolated matrix vesicles from normal cartilage. Bar represents 0.5 μM. (c) Electron micrograph of an apoptotic chondrocyte in cartilage treated with the NO donor SNP. The area indicated by the arrow is shown at higher magnification in (d). Bar represents 2 μM. (d) High magnification view of the area indicated by the arrow in (c). Bar represents 0.5 μM. NO, nitric oxide; SNP, sodium nitroprusside.

experiments where apoptosis was induced by the nitric oxide donor sodium nitroprusside or antibody to the Fas antigen [27]. Extracellular vesicles accumulate around apoptotic cells (Fig. 1). The levels of pyrophosphate produced by apoptotic bodies were increased by pretreatment of the chondrocytes with transforming growth factor-β and decreased by IL-1 interleukin-1beta (IL-1β) [27]. It has also been suggested that ACEV from primary articular chondrocytes can be generated through the autophagy pathway [28]. In normal but not in osteoarthritis chondrocytes, rapamaycin, which induces autophagy by inhibiting mTOR signaling, increased the release of ACEV that contained LC3, a marker of autophagosomes [28]. Release of ACEV was inhibited by gene knock down of caspase-3, suggesting an involvement of apoptosis-related mechanisms [28]. Thus, ACEVs include various types of extracellular vesicles that differ in mechanism of generation and apparently in molecular content.

More detailed information about calcium crystal deposition by ACEV is presented in a recent review [21▪▪]. A database of MaV proteins also provides

comprehensive information on protein components of mineralization-related MaV [29].

EXTRACELLULAR VESICLES IN COMMUNICATION AMONG JOINT TISSUES

The concept that extracellular vesicles can mediate communication among cells from different joint tissues has thus far only been tested in a limited number of examples. Exosomes from IL-1β-stimulated synoviocytes significantly upregulated matrix metalloproteinase (MMP)-13 and ADAMTS-5 expression in articular chondrocytes, and downregulated COL2A1 and ACAN compared with synoviocyte-derived exosomes that were not simulated with IL-1 [30] (Fig. 2). Migration and tube formation activity were significantly higher in human umbilical vein endothelial cells treated with the exosomes from IL-1β-stimulated synoviocytes, which also induced significantly more proteoglycan release from cartilage explants. Inflammatory cytokines, IL-6, MMP-3 and vascular endothelial growth factor in exosomes were only detectable at low level. IL-1β, tumor necrosis factor-α MMP-9 and MMP-13 were not detectable in exosomes. NanoString analysis showed that levels of 50 miRNAs were differentially expressed in exosomes from IL-1β-stimulated synoviocytes compared to nonstimulated cells [30].

EXTRACELLULAR VESICLES IN SYNOVIAL FLUID AS POTENTIAL OSTEOARTHRITIS BIOMARKERS

Extracellular vesicles are abundant in synovial fluid, and can be derived from resident cells in joint tissues and from leukocytes that infiltrate arthritis-affected joints. Synovial fluid extracellular vesicles modulate the release of chemokines and cytokines in

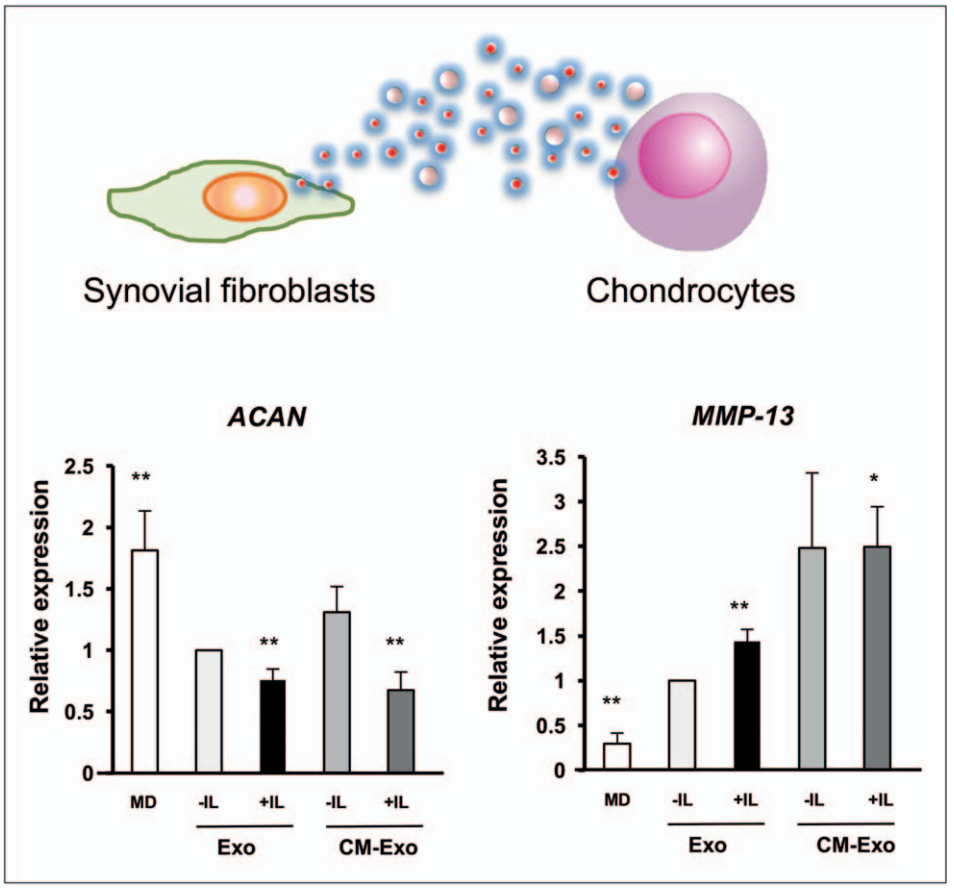

FIGURE 2. The effect of EV from IL-1β-stimulated synovial fibroblasts on normal articular chondrocytes. Articular chondrocytes were treated with fresh-DMEM, exosomes from nonstimulated synovial fibroblasts (SFBs) or exosomes from IL-1β-stimulated SFB. The expression of osteoarthritis-related genes was analyzed by real-time PCR. *MMP-13* was significantly upregulated, and *ACAN* was significantly downregulated by exosomes from IL-1β-stimulated SFB. $^{**}P < 0.01$ versus Exo –IL. EVs, extracellular vesicles; Exo –IL, exosomes from nonstimulated SFB; Exo + IL, exosomes from IL-1β-stimulated SFB; MD, DMEM with 10% FBS.

synoviocytes [31,32▪▪]. The expression patterns of miRNAs in synovial fluid of osteoarthritis were similar to miRNAs secreted by synovial tissues [33]. A recent microarray analysis of miRNAs in synovial fluid exosomes showed that in samples from female osteoarthritis patients, miR-16–2–3p was upregulated and miR-26a-5p, miR-146a-5p and miR-6821–5p were downregulated. In synovial fluid from male osteoarthritis patients, miR-6878–3p was downregulated and miR-210–5p was upregulated. Thus, synovial fluid exosomal miRNA content is altered in patients with osteoarthritis and these changes are sex specific [34▪▪]. This is the first study to analyze exosomal molecules as biomarkers in osteoarthritis. Future studies need to address the possibility of detecting joint-derived extracellular vesicles in blood and of identifying the cellular origin of extracellular vesicles. This has potential to detect tissue-specific changes as biomarkers for osteoarthritis.

EXTRACELLULAR VESICLES IN THERAPEUTIC APPLICATIONS

Mesenchymal stem cells (MSCs) have been used successfully in tissue engineering approaches to treat cartilage lesions and osteoarthritis in animal [35] and human studies [36,37]. These beneficial functions of MSC are at least in part mediated by paracrine effects of cytokines and growth factors that decreased inflammation, enhanced progenitor cell proliferation and improved tissue repair. In a mouse model of myocardial ischemia/reperfusion injury, it was first demonstrated that the protective paracrine effect was mediated by secreting exosomes [38].

Since then, additional studies have demonstrated that extracellular vesicles from MSCs have therapeutic effects [39–41]. We reported that MSC-derived extracellular vesicles promote skeletal muscle repair and bone fracture healing in mouse models through acceleration of biological functions such as angiogenesis and cell differentiation [32▪▪,42].

Exosomes can be used in therapeutic approaches, either from specific cell types such as MSC or from cells that are transfected with genes that have therapeutic potential to enrich for RNA levels for the gene of interest. Exosomes derived from synovial membrane MSC promoted chondrocyte proliferation and migration but inhibited their secretion of extracellular matrix (ECM). Wnt5a and Wnt5b carried by exosomes activated the alternative Wnt signaling pathway and enhanced proliferation and migration of chondrocytes but significantly decreased ECM secretion. We previously reported that miRNAs, in particular miRNA-140, one of the most abundant miRNAs in chondrocytes are important regulators of cartilage homeostasis [43,44]. Exosomes that were prepared from cells that were transduced with lentiviral miR-140–5p, enhanced the proliferation and migration of articular chondrocytes and reduced osteoarthritis severity in a rat model [45▪▪]. Human embryonic MSC exosomes promoted cartilage regeneration in a rat osteochondral defect model [46]. In that study, MSC exosomes accelerated neotissue filling and enhanced synthesis of type II collagen and sulphated glycosaminoglycans. By the end of 12 weeks, exosome-treated rats displayed complete restoration of cartilage and subchondral bone.

Exosomes from conditioned culture media of embryonic stem cell-derived MSC (ESC-MSC) maintained the chondrocyte phenotype by increasing collagen type II synthesis and decreasing ADAMTS5 expression in the presence of IL-1β. Intra-articular injection of ESC-MSC alleviated cartilage destruction and matrix degradation in the destabilizing the medial meniscus (DMM) model. Immunocytochemistry revealed colocalization of the exosomes and collagen type II-positive chondrocytes. Subsequent intra-articular injection of exosomes derived from ESC-MSC successfully protected against cartilage destruction in the DMM model. The exosomes from ESC-MSC exert a beneficial therapeutic effect on osteoarthritis by balancing the synthesis and degradation of chondrocyte ECM, which in turn provides a new target for osteoarthritis drug and drug-delivery system development [47]. This study demonstrated the utility of MSC exosomes as a ready-to-use and 'cell-free' therapeutic alternative to cell-based MSC therapy.

FUTURE PERSPECTIVES

The role of extracellular vesicles in joint homeostasis and osteoarthritis pathogenesis is of great interest and further research on this topic has potential implications for the discovery of novel biomarkers and therapeutic approaches. Currently, this field is at an early stage and the topic that seems most advanced is the use of extracellular vesicles as therapeutics. Key questions that need to be investigated are: regulation of the types, amounts and compositions of extracellular vesicles that are generated and released by cells; stability of extracellular vesicles in the various joint tissue environments and transport of extracellular vesicles through dense ECM structures such as in cartilage; mechanisms of recognition and internalization by cells; the role of extracellular vesicles in joint homeostasis and pathogenesis; markers of extracellular vesicles

in synovial fluid or blood that allow tracking their cellular origin and thus profiling the status of these cells.

CONCLUSION

Extracellular vesicles are present in articular cartilage and synovial fluid and represent a heterogeneous mixture that varies in regard to mechanism of generation and molecular content. Synovial fluid extracellular vesicles are potential new osteoarthritis biomarkers. Composition of extracellular vesicles from osteoarthritis cartilage appears to be altered and may contribute to abnormal mineral and crystal deposition. Extracellular vesicles are released from synovial fibroblasts and affect gene expression in chondrocytes. The pattern of mRNAs and miRNAs in extracellular vesicles can be altered by stimulation or gene transduction of cells and thus be designed to specifically change the function of target cells for therapeutic use.

Acknowledgements

None.

Financial support and sponsorship

This work was supported by NIH grant AG 007996 to M.L. and MEXT/JPS KAKENHI fund for the Promotion of Joint International Research 15KK0308 to S.M.

Conflicts of interest

There are no conflicts of interest.

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